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13. ABSTRACT (Maximum 200 Words) Ataxia-Telangiectasia (A-T) is an autosomal recessive disease with a complex phenotype including increased risk for cancer and radiation sensitivity. Moreover, carriers are suspected to be prone to breast cancer. We have studied the role of ATM, the product of the gene mutated in A-T. We have used cell-free system derived from Xenopus eggs to obtain novel insights on the biochemical function of ATM. We have cloned the Xenopus homologue of ATM, X-ATM and generated specific antibodies to study its function. We have described X-ATM temporal expression pattern during the cell cycle as well as its spatial distribution during development. We have shown that X-ATM is present in high molecular weight complexes. We have then demonstrated the role of X-ATM in checkpoint signaling. We have reconstituted a cell-free system that recapitulates the cellular phenotype of A-T called radio-resistant DNA synthesis (RDS). We have shown that ATM is essential to signal following DNA double-strand breaks (DSBs). This ATM-dependent checkpoint leads to the inhibition of DNA replication initiation. We have also established that DSBs can directly activate X-ATM protein kinase in cell-free extracts. Finally, we have analyzed the functional interactions between X-ATM and X-Mre11 a protein essential for repair that displays a similar phenotype to A-T, when mutated.			
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INTRODUCTION

Ataxia Telangiectasia (A-T) is an autosomal recessive disease that displays a complex phenotype (1, 2). Patients exhibit a progressive cerebellar ataxia, in addition to severe immune deficiencies, gonadal atrophy, telangiectases, increased risk for cancer, particularly lymphomas, and radiation sensitivity. Additionally, carriers are suspected to be prone to other cancers including breast cancer (3-6).

Cells from A-T patients show increased radiosensitivity to ionizing radiation (7), increased chromosomal loss and breakage, and abnormal telomere morphology (8, 9). Furthermore, these cells are defective in cell cycle checkpoints in G1, S and G2 phases of the cell cycle (10-12). A striking cellular phenotype of A-T is the inability to prevent DNA replication following DNA damage also called Radioresistant DNA Synthesis (RDS). Although complex, the cellular phenotype of A-T points to a defect in handling DNA breaks formed either following damage or subsequent to normal physiological processes such as meiotic recombination and the maturation of the immune system.

Cells respond to DNA damage by activating checkpoint pathways that delay progression through the cell cycle (13, 14). ATM is a protein kinase that functions in the checkpoint pathways activated by DNA damage. ATM is a central component of the DNA damage response. This response is impaired in cancers, breast cancer in particular. Indeed, the known substrates of ATM include p53, a gene mutated in sporadic breast cancers and BRCA1, the major susceptibility gene for breast and ovarian cancer (15-17).

Xenopus laevis is a powerful model system for both biochemical studies of cell cycle and checkpoint regulation as well as for developmental studies. We decided to use Xenopus as a novel model system to study both the biochemical role of ATM and its function during development. In this final report we summarize our 4-years project on the cloning, characterization and functional studies on the Xenopus homologue of ATM (X-ATM). To date, this work has lead to 5 publications (18-22) as well as some additional work in preparation.

The major objective of this proposal was to study the function of the ATM protein in a simple system in which biochemical analysis is a very powerful tool.

BODY

Cloning of X-ATM, the Xenopus homologue of ATM

Using a PCR based strategy with degenerated oligonucleotides we have isolated a DNA fragment for Xenopus ATM (X-ATM) from the XTC cell line. We have then screened a total of 6 cDNA libraries from different developmental stages and from a Xenopus cell line. None of these libraries contained a full-length X-ATM cDNA, therefore we have subsequently used 5' RACE to obtain the full-length cDNA. X-ATM is highly homologous to the human protein even in the central portion of the protein, away from the kinase domain, the business end of the molecule (see appendix, Item #1).

Generation of specific antibodies against X-ATM

We have raised polyclonal antibodies against recombinant X-ATM that are highly specific for the ATM protein and recognize a single polypeptide of 370-kDa in oocytes, embryos, egg extracts and a Xenopus cell line (see appendix, Items #1,2,3 and 4).

Spatial and temporal expression of X-ATM during development

Using a combination of Northern blot analysis and RNase protection assay we have studied the temporal expression of Xenopus ATM during early development (See appendix, Item #1). We found that the mRNA is expressed maternally (zygotic transcription in Xenopus does not start until stage 9), suggesting that ATM could play a role in early development. We have also characterized the spatial expression during development. We have analyzed the patterns of expression of both X-

ATM mRNA and protein. Our findings show that both mRNA and proteins switch from a uniform expression during early development to specific patterns as differentiation and organogenesis proceeds. In particular we found high levels of expression in both the developing somites and the developing eyes. A detailed description of our findings can be found in the appendices in an article that was recently published (see appendix, Item#2).

Expression of X-ATM during the cell cycle

Since ATM has been proposed to be involved in the cell cycle checkpoint response following DNA damage, we have assessed whether X-ATM protein levels fluctuated throughout the cell cycle. Xenopus "cycling extracts" provide the ideal material to perform such analysis as these cell-free extracts undergo genuine cell cycle transition *in vitro* in a synchronous manner. The levels of X-ATM do not change throughout the cell cycle. Furthermore we have followed the expression pattern of X-ATM protein during the highly synchronous meiotic of Xenopus laevis (see appendix, Item #1). We also have followed the expression of the protein during oogenesis and found that X-ATM was expressed maternally as early as stage II pre-vitellogenic oocytes. Subcellular fractionation showed that the protein was nuclear in both the female and male germlines. In addition, we did not observe any change in the level or mobility of X-ATM protein following γ -irradiation of embryos (see appendix, Item #1).

X-ATM forms high molecular weight complexes

Using non denaturing gradient polyacrylamide gels followed by western blotting we showed that the native X-ATM protein migrates as a monomer of about 350 KDa as well as a higher molecular weight complex of about 500 KDa.

Reconstitution of an ATM-dependent DNA damage checkpoint *in vitro*

We have developed a cell-free system that reconstitutes an ATM-dependent DNA damage checkpoint *in vitro*. This is the first cell-free system in which to study DNA damage checkpoint. It allows the detailed biochemical characterization of the signaling pathways that lead to cell cycle arrest following DNA damage. This will help understand how these pathways are altered in the case of cancer and breast cancer in particular and will provide critical information to design successful therapies. The detailed description of the cell-free system and of the checkpoint pathway we have discovered can be found in the appendix (Item #3).

This pathway is activated *in vitro* by double-strand breaks DSBs, the most harmful form of DNA damage. Signaling is dependent on ATM and is completely abolished by antibodies specific for X-ATM. Signaling leads to the inhibition of Cdk2/cyclinE activity. The inhibition of Cdk2 is due to the phosphorylation of tyrosine residue 15. The inhibition of Cdk2 prevents the loading of cdc45 on the pre-replicative complex, preventing DNA replication initiation.

In vitro activation of ATM and DNA damage sensitive protein kinases

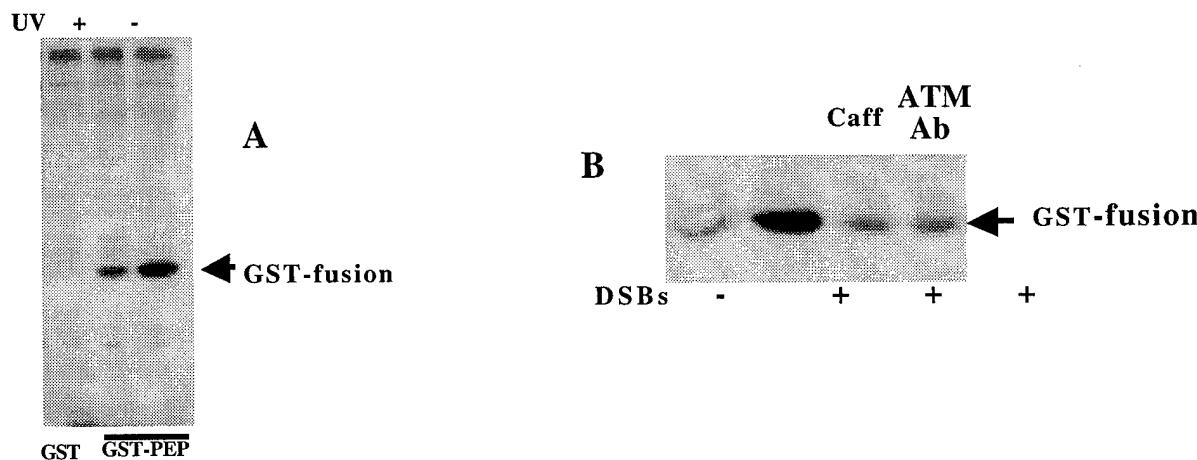


Figure 1: A rapid assay for DNA damage-activated protein kinases. A) UV-induced DNA damage. Xenopus high speed supernatant (HSS) derived from interphase extract is supplemented with control plasmid (-) or UV-irradiated plasmid (+). Plasmid is irradiated with 500 J/m² creating approximately 5 CPD/kb (23). After 20 minutes of incubation, 2 μ l aliquot of extract is added to 18 μ l of kinase assay buffer (24) containing 2mg/ml of purified GST (control) or GST protein fused with the BRCA1 peptide containing a "SQ" site (see text for detail). The kinase reaction is incubated for 20 minutes and processed for PAGE and autoradiography. UV damage triggers the induction of protein kinases that can phosphorylate the GST-fused peptide (GST-PEP). **B) DSBs.** The assay is similar as in A), DSBs-containing DNA (10 ng/ μ l) is added instead of UV-irradiated plasmid. DSBs trigger protein kinase(s) that can phosphorylate the GST-Peptide (compare lane 1 and 2). This induced phosphorylation is quantitatively inhibited by 5mM caffeine (lane 3) and by X-ATM antibodies (lane 4).

We have recently designed a rapid assay to monitor the DNA damage-induced activation of ATM protein kinase *in vitro* (Figure 1 and manuscript in preparation). In this assay we used a reporter substrate that was designed from the sequence of a known ATM phosphorylation site in the BRCA1 protein (15). This assay will allow us to further study the mechanisms of DNA damage recognition which are very poorly characterized.

The essential function of Mre11

Mre11 interacts physically and functionally with Mre11, a gene that is essential for DNA double-strand breaks repair. We have studied the function of Mre11 in the cell-free system described above. We have cloned the Xenopus homologue of Mre11, X-Mre11. We have found that X-Mre11 is activated by DNA double-strand breaks. Unlike X-ATM, X-Mre11 is not essential for the DNA damage checkpoint that prevents initiation of DNA replication. X-Mre11 is important for DNA replication as it monitors and repairs DNA breaks occurring as a consequence of normal DNA replication. This work was recently published in a manuscript that can be found in the appendix (Item #5).

KEY RESEARCH ACCOMPLISHMENTS

- Cloning and sequencing of X-ATM, the Xenopus homologue of ATM, the gene mutated in Ataxia-telangiectasia (A-T).
- Expression of X-ATM protein fragments in *E. coli*, purification of the expressed proteins.
- Generation of specific antibodies against X-ATM protein.
- Description of X-ATM protein and mRNA spatio-temporal patterns of expression.
- Establishment of a Cell-free system that recapitulates a DNA damage checkpoint.
- Unravel a novel DNA damage checkpoint pathway that is ATM-dependent.
- Elucidate the functional relationships between ATM and MRE11, 2 genes mutated in cancer-prone syndromes

REPORTABLE OUTCOMES

Manuscripts:

Robertson, K., C. Hensey, and **J. Gautier**, Isolation and characterization of Xenopus ATM (X-ATM): expression, localization, and complex formation during oogenesis and early development. *Oncogene*, 1999. **18**(50): p. 7070-9.

- Hensey, C., K. Robertson, and **J. Gautier**, Expression and Subcellular localization of X-ATM During Early Xenopus Development. *Development, Genes and evolution*, 2000. **210**: p. 467-469.
- Costanzo, V., Robertson, R., Ying, C., Kim, K., Avvedimento, E., Gottesman, M., Grieco, D., and **Gautier, J.** Reconstitution of an ATM-dependent checkpoint that inhibits chromosomal DNA replication following DNA damage. *Molecular Cell*, 2000. **6**: p. 649-649.
- Greenwood J., Costanzo V., Robertson K., Hensey C. and **Gautier J.** (2001). Responses to DNA damage in Xenopus: cell death or cell cycle arrest. *Novartis Found Symp*. 237:221-30; discussion 230-4.
- Costanzo, V., Robertson K., Bibikova M., Kim E., Grieco D., Gottesman M., Carroll D. and **Gautier J.** (2001). Mre11 protein complex prevents double-strand break accumulation during chromosomal DNA replication. *Molecular Cell*, 8, 137-147.

Abstracts and presentations:

ATM International Conference, Las Vegas, 1999.

DNA Replication meeting, Cold Spring Harbor, September 1999.

Novartis Conference: "Cell cycle and development". London, April 2000.

Era of Hope Meeting. Atlanta, June 2000.

8th International Xenopus Conference. Estes Park (CO), August 2000.

DNA Replication Meeting, Salk Institute. San Diego, September, 2000.

AACR (American Association of Cancer Research). New Orleans, April 2001.

DNA Replication meeting, Cold Spring Harbor, September 2001.

Keystone Symposium on DNA Replication and DNA Replication, Utah, January 2002.

ATM International Conference, 2002.

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CONCLUSIONS

In summary, we have accomplished most of the tasks proposed in the original SOW. Objective #1 has been completed and the results have been reported in 2 publications (20, 21). Objective #2 has also been completed, although we opted for a different strategy to identify ATM-associated proteins, as explained in last year's report. This work was published as well (18, 21). Finally, we have studied in detail the role of Mre11, an essential gene that interacts functionally and physically with ATM (18, 19). Our approach has been very successful and has allowed several breakthroughs in the field of the DNA damage response. The findings we originally reported in Xenopus cell-free extracts (18, 19) have been now extended by others to mammalian systems, thus demonstrating the power of our *in vitro* approaches.

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APPENDIX:

Item #1: Robertson, K., C. Hensey, and **J. Gautier**. *Oncogene*, 1999. **18**(50): p. 7070-9.

Item #2: Hensey, C., K. Robertson, and **J. Gautier**. *Development, Genes and evolution* 2000. **210**: p. 467-469.

Item #3: Costanzo, V., Robertson, R., Ying, C., Kim, K., Avvedimento, E., Gottesman, M., Grieco, D., and **Gautier, J.** *Molecular Cell*, 2000. **6**: p. 649-649.

Item #4: Greenwood J., Costanzo V., Robertson K., Hensey C. and **Gautier J.** (2001). Novartis Found Symp. 237:221-30; discussion 230-4.

Item #5: Costanzo, V., Robertson K., Bibikova M., Kim E., Grieco D., Gottesman M., Carroll D. and **Gautier J.** (2001). *Molecular Cell*, 8, 137-147.

Item #6: Updated CV

Isolation and characterization of *Xenopus* ATM (X-ATM): expression, localization, and complex formation during oogenesis and early development

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ATM, the gene product mutated in Ataxia Telangiectasia (A-T) encodes a 350-kDa protein involved in the regulation of several cellular responses to DNA breaks. We used a degenerate PCR-based strategy to isolate a partial clone of X-ATM, the *Xenopus* homologue of human ATM. Sequence analysis confirmed that the clone was most closely related to human ATM. *Xenopus* ATM protein (X-ATM) is 85% identical to human ATM within the kinase domain and 71% identical over the carboxyl-terminal half of the protein. Polyclonal antibodies raised against recombinant X-ATM are highly specific for the ATM protein and recognize a single polypeptide of 370-kDa in oocytes, embryos, egg extracts and a *Xenopus* cell line. We found that X-ATM was expressed maternally in eggs and as early as stage II pre-vitellogenic oocytes, and the protein and mRNA were present at relatively constant levels throughout development. Subcellular fractionation showed that the protein was nuclear in both the female and male germlines. The level of X-ATM protein did not change throughout the meiotic divisions or the synchronous mitotic cycles of cleavage stage embryos. In addition, we did not observe any change in the level or mobility of X-ATM protein following γ -irradiation of embryos. Finally, we also demonstrated that X-ATM was present in a high molecular weight complex of approximately 500 kDa containing the X-ATM protein and other, as yet unidentified component(s).

Keywords: *xenopus*; ATM; development; cell cycle; checkpoints

Introduction

Ataxia Telangiectasia (A-T) is an autosomal recessive disease which displays a complex phenotype (Boder and Sedgwick, 1970; Shiloh, 1998). Patients exhibit a progressive cerebellar ataxia, in addition to severe immune deficiencies, gonadal atrophy, telangiectases, increased risk for cancer, particularly lymphomas, and radiation sensitivity. Additionally, carriers are suspected to be prone to other cancers including breast cancer (Athma *et al.*, 1996; Chen *et al.*, 1998; Stankovic *et al.*, 1999; Swift *et al.*, 1990; Yuille and Coignet, 1998).

Cells from A-T patients show increased radiosensitivity to ionizing radiation (Lavin and Shiloh, 1997), increased chromosomal loss and breakage, and abnormal telomere morphology (Smilenov *et al.*, 1997; Vaziri *et al.*, 1997). Furthermore, these cells are defective in cell cycle checkpoints in G1, S and G2 phases of the cell cycle (Beamish *et al.*, 1996; Hoekstra, 1997; Meyn, 1995). Although complex, the cellular phenotype of A-T points to a defect in handling DNA breaks formed either following damage or subsequent to normal physiological processes such as meiotic recombination and the maturation of the immune system.

The identification of a single mutated gene called ATM (Ataxia Telangiectasia Mutated) as the molecular basis for the phenotype has allowed a better understanding of both ATM function and the A-T pleiotropic phenotypes (Savitsky *et al.*, 1995; Taylor, 1998). ATM is a nuclear phosphoprotein (Chen and Lee, 1996; Scott *et al.*, 1998) and is a Serine/Threonine protein kinase for which the c-abl proto-oncogene, the replication protein RPA and the p53 tumor-suppressor gene have been identified as substrates *in vitro* (Banin *et al.*, 1998; Baskaran *et al.*, 1997; Canman *et al.*, 1998; Gately *et al.*, 1998; Khanna *et al.*, 1998; Nakagawa *et al.*, 1999; Shafman *et al.*, 1997). The identification of these potential substrates have helped place ATM in a signal transduction pathway in which it could function in a cell cycle checkpoint through c-abl and p53.

Cells respond to DNA damage by activating checkpoint pathways that delay progression through the cell cycle (Hensey and Gautier, 1995). ATM functions in the checkpoint pathway activated by DNA damage. It has been shown that p53 is phosphorylated in an ATM-dependent fashion following DNA damage suggesting that p53 could be a direct substrate for the ATM protein kinase (Banin *et al.*, 1998; Canman *et al.*, 1998). Activation of p53 following DNA damage also involves dephosphorylation events that are dependent upon ATM activity (Waterman *et al.*, 1998). In addition, studies in yeast and in mammalian cells also suggest that ATM might function as an upstream regulator of the Chk1 and Chk2 kinases (Brown *et al.*, 1999; Chen *et al.*, 1999; Matsuoka *et al.*, 1998). However, the exact *in vivo* biochemical function of the ATM protein and its physiological substrates still remain elusive. In particular, the gap between our *in vitro* biochemical knowledge of the protein and its relationship with both cellular and patient phenotypes still awaits investigation.

A mouse model for ATM deficiency was created in several laboratories by specific germline inactivation of

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the ATM gene (Barlow *et al.*, 1996; Elson *et al.*, 1996; Herzog *et al.*, 1998; Xu *et al.*, 1996; Xu and Baltimore, 1996). Fibroblasts isolated from ATM^{-/-} mice display similar cellular phenotypes to those observed in cells from A-T patients. As is the case with A-T patients, ATM deficient mice display a variety of growth defects, meiotic defects, immunological abnormalities, acute radiation sensitivity and cancer predisposition, confirming the pleiotropic roles of ATM.

Xenopus laevis is a powerful model system for both biochemical studies of cell cycle and checkpoint regulation as well as for developmental studies. We decided to use *Xenopus* as a novel model system to study both the biochemical role of ATM and its function during development. The influence of DNA replication inhibition upon cell cycle progression (DNA replication or S phase checkpoint) has been studied in detail in *Xenopus* egg extracts. First, it has been shown that threshold inhibition of DNA replication can elicit cell cycle arrest in egg extracts and prevent entry into mitosis (Dasso and Newport, 1990). Furthermore, both cdc25 and wee1 have been identified as modulators of this checkpoint. Upstream of cdc25, the protein kinase Chk1 and the 14-3-3 adapter protein have also been shown to be essential for cell cycle arrest (Kumagai *et al.*, 1998a,b).

To enable such studies we have obtained a partial clone of the *Xenopus* homolog of the ATM protein. We present its detailed expression patterns during the meiotic and the mitotic cell cycles as well as during development. We show that X-ATM is a maternal protein expressed as early as stage II oocytes and that ATM is exclusively nuclear in the germline. Finally, we present evidence that ATM exists in a large molecular weight complex in extracts derived from eggs.

Results

Isolation of a partial clone of the *Xenopus* homolog of ATM

A protein alignment of the putative kinase domain of human ATM, *S. pombe* rad3, human ATR, *S. cerevisiae* TEL1, and *D. melanogaster* mei41 was used to identify regions of homology within the kinase domain of ATM and TEL1, that were not present in ATR, rad3 or mei41. Four amino acid stretches were identified and used to design degenerate oligonucleotides (see Materials and methods, Figure 1b). A 480 bp cDNA fragment was isolated by RT-PCR (Figure 1a) and sequencing of the fragment confirmed that it was most closely related to human ATM (Figure 1b). Using library screening, ATM-specific library construction, and 5'RACE (Figure 1a) additional sequence corresponding to approximately 50% of the predicted *Xenopus* ATM ORF was obtained.

An alignment of the carboxyl-terminal half of X-ATM with human ATM is shown in Figure 1b. ATM is highly conserved between *Xenopus* and human with an overall identity of 71% in the region we have sequenced. Within the kinase domain the identity is 85% while the region upstream of the kinase domain (amino acids 1–1100 of X-ATM) shows 66% identity

to the human protein. In contrast, when this region from X-ATM (a.a. 1–1100) is aligned with *S. c. TEL1*, *S. p. rad3*, *D. m. mei41* or *H. s. ATR*, only short stretches of weak homology (ranging from 20–24%) are detected.

Characterization of X-ATM antibodies

Using an *E. coli* expression vector, we produced a 20-kDa fragment of the X-ATM cDNA corresponding to the region immediately 5' of the protein kinase domain (PQE60/TBH6H of Figure 1a). We chose this region as it had been previously shown to elicit a good immune response for human ATM (Brown *et al.*, 1997). Additionally, we wanted to avoid raising antibodies against the conserved kinase domain which could potentially cross-react with other members of the ATM/PI3 kinase family. This 20-kDa polypeptide was expressed in *E. coli* and was used as an antigen for antibody production in rabbits (see Materials and methods).

Programmed reticulocyte lysate containing the 65-kDa carboxyl terminal portion of X-ATM (T7/TBH-XTc of Figure 1a) was used to screen the different antisera. Sera from both rabbits showed strong cross-reactivity with the translated product (data not shown) and recognized a protein of approximately 370-kDa in *Xenopus* egg extracts (Figure 2a, lanes 1, 2). Due to the presence of a 210-kDa cross-reacting polypeptide in serum A (Figure 2a, lane 1), we used serum B for the remaining experiments. Preincubation of the antiserum with the 20-kDa polypeptide used for immunization eliminated the signal, confirming the identity of the protein recognized by the antiserum (data not shown). Moreover, we were able to immunoaffinity purify the X-ATM antibody from rabbit B using the 20-kDa polypeptide coupled to agarose. This purified antibody, which was used in all further experiments, detected a single polypeptide in *Xenopus* egg extracts that migrated slightly slower than human ATM (compare lanes 3 and 4, Figure 2a). It also immunoprecipitated a fraction of the X-ATM protein from extracts as shown in Figure 2b (lane 2).

Expression of X-ATM during early development

Little is known about ATM expression during the early phases of vertebrate development. We therefore examined the temporal expression of both X-ATM mRNA and protein between fertilization and the swimming tadpole stage in *Xenopus*. Figure 3a shows an RNase protection assay for the XTC cell line (Pudney *et al.*, 1973) and the indicated stages of development. X-ATM mRNA was detected in unfertilized eggs and expressed at relatively constant levels throughout cleavage, gastrulation, neurulation and the tadpole stage, although we consistently detected higher X-ATM mRNA levels in unfertilized eggs. Since transcription of zygotic genes does not start until the midblastula transition (St. 8–9), this demonstrates that X-ATM mRNA is maternally inherited. The temporal expression of X-ATM protein during similar developmental stages is shown in Figure 3b. The protein is expressed maternally and throughout development where we observe a single cross-reacting polypeptide at all stages. Some varia-

bility in the X-ATM protein amounts was detected, particularly during neurulation. This might reflect developmentally regulated changes in the amount of X-ATM. Alternatively, this could also be due to the subcellular localization of the protein leading to different yields following embryonic extract preparation. We estimated the concentration of X-ATM to be 200 nM in egg extracts and 40 nM in unfertilized eggs (see Materials and methods).

Subcellular localization of the ATM protein in the germline

X-ATM is present in egg extracts and in unfertilized eggs (Figures 2a and 3b). We examined whether the protein was already present in the oocyte as opposed to being synthesized during meiotic maturation as has been described for some proteins (Sagata *et al.*, 1988). *Xenopus* oocytes were manually dissected from pieces of ovary and protein extracts were made at the stages of oogenesis indicated (Dumont, 1972). X-ATM is already present in previtellogenic oocytes (stage II) and persists throughout oogenesis (Figure 4a). Although the concentration of X-ATM per mass of tissue does not vary significantly throughout oogenesis, the mass per oocyte increases dramatically since the volume of the oocytes increases by a factor of 10³ between stages I–II and stage VI.

Stage VI *Xenopus* oocytes contain a large (200 µm diameter) nucleus, known as the germinal vesicle (GV), which can be used to store nuclear proteins important for later development (Dreyer and Hausen, 1983). The GV can be isolated surgically from its surrounding cytoplasm, allowing the preparation of pure cytoplasmic and nuclear fractions from oocytes (Ford and Gurdon, 1977). We demonstrate that X-ATM is entirely nuclear in stage VI oocytes (Figure 4b). Moreover, using either demembranated sperm nuclei, or a nuclear extract prepared from these nuclei following their incubation in extracts (Walter *et al.*, 1998), we show that X-ATM is also nuclear in the male germline.

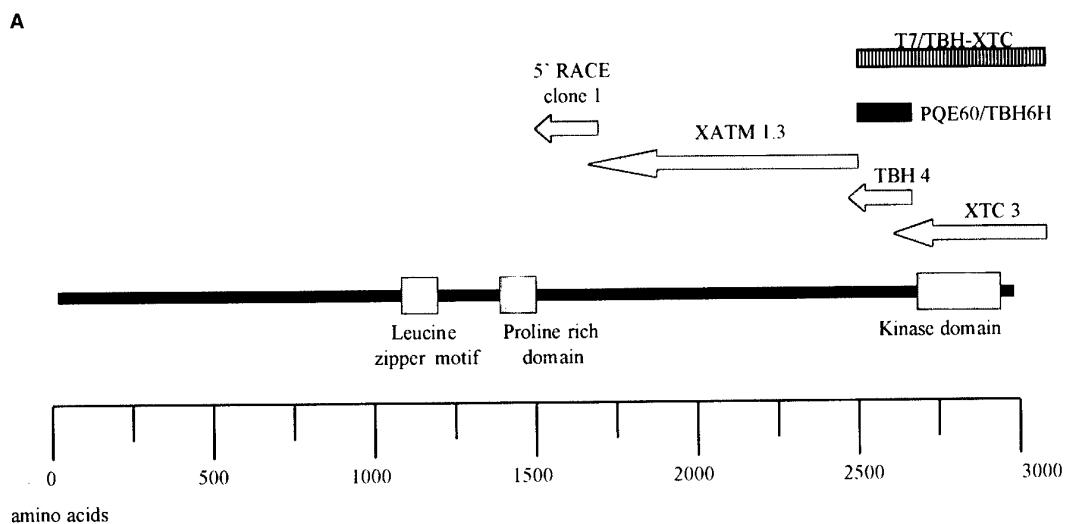
Expression of X-ATM protein throughout the meiotic and mitotic cell cycles

In *Xenopus* oocytes the highly synchronous meiotic cell cycle is easily studied *in vitro*, where progesterone induced oocyte maturation culminates in the completion of meiosis I and the progression to metaphase of meiosis II. To determine whether X-ATM protein levels fluctuated during the meiotic cell cycle, we performed Western blots on protein samples from oocytes synchronously undergoing meiosis. X-ATM protein levels remained constant throughout meiosis (Figure 5a). In this experiment, prophase-arrested oocytes were induced to undergo meiotic maturation *in vitro* by progesterone. Nuclear envelope breakdown took place at 3.5 h, meiosis I occurred at 6 h and the oocytes were arrested in metaphase of meiosis II by 9 h.

Next, we followed X-ATM levels throughout the mitotic cell cycle which oscillations were assessed by following cdc2/cyclinB activity. The naturally synchronous cell divisions of the early embryo allow the preparation of cell free extracts that undergo cell cycle oscillations *in vitro*. Using such extracts we examined X-ATM protein levels throughout these highly synchronized cell cycles. ATM is expressed throughout the cell cycle and its levels remain constant over the course of two cell cycles (Figure 5b). Additionally, there was no change in the electrophoretic mobility of X-ATM.

X-ATM protein in γ -irradiated eggs

In *Xenopus*, irradiation of cleavage stage embryos leads to massive and synchronous apoptosis at the onset of gastrulation (St. 10.5) (Anderson *et al.*, 1997; Hensey and Gautier, 1997). To assess whether this response correlated with changes in the amounts or the electrophoretic mobility of X-ATM, we compare the levels of X-ATM in protein samples from untreated and γ -irradiated embryos between stage 8 and 10.5. X-ATM protein levels were similar in control and irradiated embryos. In addition no change in the mobility of the protein was detected (Figure 6).



X-ATM	DVSTRSLLC LDLLHRVCR AYTHCEDALE CHHHVIVGSL	40	X-ATM	MPOIKQYNS QLGVSEWOLE EAOIFWDKEE PSLARBLKQ	840
H-ATM	DVSTRSLSC CDLLSQVCQ AVTYCKDALE NHHLHVIVGTL	1520	H-ATM	IPQIKQYNSV SCGVSEWOLE EAQVFWAKKE QSLALSLIKQ	2310
Consensus	DVS.RS..LC .DLL..VC.. AVT.C.DAHL .H..HVIVG.L		Consensus	P QIKQYNSV ..GVSSEWOLE EAQ .F.W.K.B .SLA..ILKQ	
X-ATM	IPLANSQESI QEKGVC DLLNF LVLENKDNEN LYHTIKLLDP	80	X-ATM	MINKLEIKSF EVENDSRLR LYAESLRLCG KWLAETCLES	880
H-ATM	IPLVYEQVEV QKQVLDLKKY LVIDNKDNEN LYITIKLLDP	1560	H-ATM	MIKKLDASC -AANNPSLKL TYTECLRVCG NWLAETCLEN	2348
Consensus	IPL...Q...Q..V.DLL.. LVI.NKDNEV LY.TIKLLDP		Consensus	M I.KL.....N..L.L .Y.E.LR.CG .WLAETCLEN.	
X-ATM	FPPDPLFKNL RQAHQKIKY KGPFSLLKEI QNPLSVSVD	120	X-ATM	PTVIMONYLE KAVEFAG-Y SGAGEKLOQG RMKAFLSLAR	919
H-ATM	FPPDHVVFKL RITQKQKIKY RGPFSLLKEI NHPLSVSVD	1600	H-ATM	PAVIMOTYLE KAVEVAGNDQ GESSDELNRG KMKAFSLSLAR	2388
Consensus	FPP..F.K.L ..QKIKY .GPFSLL.BI ..FLSVS.V.D		Consensus	P .VIMQ.YLE KAVE.AG.Y.L..G .MKAFSLSLAR	
X-ATM	SLPLTRBLGL NDLRKOLEQH KQDQIKELVR COGTPQDSV	160	X-ATM	PSDAQYQRID NYMKSSPEEN KQALLRKAKE EVGLIKQHVK	959
H-ATM	SLPLTRBLGL KDLRQLELH KQDMVDMRA SQDNPDQGIM	1640	H-ATM	PSDTQYQRIE NYMKSSPEEN KQALLRKAKE EVGLLREHKKI	2428
Consensus	SLPLTRBLGL .DLR.QLE.H KQDQ...R .Q..PQD...		Consensus	PS DAQYQRID NY MKSSPEEN KQ ALLRKAKE EV GLIKQHVK	
X-ATM	ASLVNLLQL SKNAHVHQNSG KFVLEAVGSC LGELGPIDFS	200	X-ATM	QNNRYTVKVE RELQLDECAI LALREDRKR KCKAVENYI	999
H-ATM	VKLVNLQL SKMAINHTGE KEVLEAVGSC LGEVGPIDFS	1680	H-ATM	QNNRYTVKVE RELELDELAL RALKEDRKR KCKAVENYI	2468
Consensus	..L.VVNLQL SK.A..... KEVLEAVGSC LG.E.GPIDFS		Consensus	Q .NNRYTVKVE .REL.LDE.A .AL.E.DRKR KCKAVENYI.	
X-ATM	NIALQHQKKD SYVLKADKVF EKEKLOCVLW MLTLINNALT	240	X-ATM	CLVSGEEHDM WIFRLCSLWL ENSAVSDVNS MMRQDAQKIP	1039
H-ATM	TIAIQHSKDA S-YTKALKLF EDKELOWTPI MLTLVNNLT	1719	H-ATM	CLLSEGEHDM WVFRLCSSLW ENSCVSEVNG MMKRQDGKIP	2508
Consensus	.IA.Q..K.. S.Y.KA.K.F B.KBLQ...MLT..NN.L.		Consensus	CL VSGEEHDM W .PRLCSLWL E NS.VS.VN. MM..D..KIP	
X-ATM	DHCIOVRSAA ATCLKNILAT KTGAMWEAC KDRGEPMILY	280	X-ATM	SHKFLPLMYQ LAARMGKTM GNPGFHDVNL NLIGRISMDH	1079
H-ATM	EDCVVRSA VTCILKNILAT KTCHSFWEIY KMTTIDPMILY	1759	H-ATM	TYKFLPLMYQ LAARMGKTM GGGLGHEVNL NLISRISMDH	2548
Consensus	..C..VRSAA .TCLKNILAT .PHE..K..PML.Y		Consensus	..K FLPLMYQ LA ARMGKTM M G..GPF.VLN N LI.RISMDH	
X-ATM	LQPPFRAPKK PLEVEEBIQRE GALESLDDPG IWIPQENHE	320	X-ATM	PHHTLFILILA LANANKDDQL MKAEEAVKRSR LTKNAPKQIS	1119
H-ATM	LQPFTRSRKK PLEVPRFDKE NPFFEGLDDIN IWIPLSENHD	1799	H-ATM	PHHTLFILILA LANANRDEFL TKPEVARRSR ITKVNPKOSS	2588
Consensus	LQPF..R..KK PLEV....B ...B.LDD.. WIP..ENH.		Consensus	PH HTLFILILA L ANAN.D..L .K.E...RSR .TKN.PKQ.S	
X-ATM	SWIKHLTCTL LESGGVKSEV LLLLKPMCEV KADPCQAVP	360	X-ATM	QLDKERMEA RHIVDTIKKR RTDMVRDVER LCDAYITLAN	1159
H-ATM	IWIKLTCAF LDSGGTKCEJ LQLLKPMCEV KTDPCQTVLP	1839	H-ATM	QLDDETRPEA NRIICTIRSR RPQMVRVSVA LCDAYIILAN	2628
Consensus	.WIK.LTC.. L.SGG.K.E. L.LKPMCBV K.DPCQ.V.P		Consensus	QL DKERMEA R HI V DTIKKR R TD M VRDVER L CDAYITLAN	
X-ATM	YIVHNLLNLD SNOJWTRLILS KNVQRFPTSC CRSLPSSRS	400	X-ATM	MDANQKSR NAIPIPSDQP ITKLNKLHDV VIPTMEIKVD	1199
H-ATM	YIHDILLDQD TNESWRNLIS THVQGFTTSC LRHQSQT	1879	H-ATM	LDATQWKTOR KGINTLQD ITKLNKLHDV VIPTMEIKVD	2668
Consensus	..B.ILL.D ..N..WR.LLS ..VQ..FFTSC ..R..SRS		Consensus	DA QKSR .QR ..I..IP.DOP IT KL..L.DV V .PTMEIKVD	
X-ATM	ATPASSDSES EGVARGAVIDI ASRRTMLJTMV EHRLKORPV	440	X-ATM	PSGEYENLVT IVFSKPEFRL AGGLNLPKII DCVGSDDGKER	1239
H-ATM	TTPANLDSES EHFFRCCLDK KSQRTMLAVV DMYRDKRPS	1919	H-ATM	HTGEYENLVT IQSKFAEFL AGGVNLPKII DCVGSDDGKER	2708
Consensus	.TPA..DSES B...R..D.. S.RTML..V ..R.Q.RP.		Consensus	..G Y..NLT I .SPK.EFRL AG .NLPKII DC VGSDDGKER	
X-ATM	SGTAFDDNFW LDNLNLYEAVM AVQSCAAHPT ALLYSEIYTD	480	X-ATM	ROLVKGODDL RQDAVMQOQF QCNCNTLQORN SETRKRKLTI	1279
H-ATM	SGTIPNDFAW LDNLNLYEAVM VAQSCAAHPT ALLYABIYAD	1959	H-ATM	ROLVKGRDDL RQDAVMQOQF QCNCNTLQORN TETRKRKLTI	2748
Consensus	SGT.F.D.FW LDNLNLYEAVM ..QSCAAHPT ALLY.BIY.D		Consensus	R OLVKGODDL R QDAVMQOQF QC NCNTLQORN E TRKRKLTI	
X-ATM	KVKQDGBORT SANRSNARK LKFEEGSQT DITGLSEKSK	520	X-ATM	RRYKVVPPLSH RSGVLEWCTG TVPIGEYLVN DKDGAHKRYR	1319
H-ATM	KKSMDQD-- KRS LAFEEGSQT TISSLSEKSK	1990	H-ATM	CTYKVVPPLSQ RSGVLEWCTG TVPIGEYLVN NEQGAHKRYR	2788
Consensus	K...D..D.....K.R. F.BEGSQ.. .I..LSEKSK		Consensus	..Y KVVPPLSH R SGVLEWCTG T PIGEYLVN ..DGAHKRYR	
X-ATM	EETG1SLQDL LMDIYRSIGE PDSLYCGGG KMLHPLARIR	560	X-ATM	PGDYGSLQOQ RKMMEVQRGR FEEKYQMPIN VCENPRPVFR	1359
H-ATM	EETG1SLQDL LLEIYRSIGE PDSLYCGGG KMLQPITRL	2030	H-ATM	PNPDSAFQOQ KKMMEVQRKS FEEKYEVFM VQCNQPVFR	2828
Consensus	EETG1SLQDL L..IYRSIGE PDSLYCGGG KML.P..R.R		Consensus	P .D...QOQ ..KMMEVQ... FEEKY..F.. V.C..NF..PVFR	
X-ATM	TYEHEAKWKG ALVTYDLEMN LPPVTRQAGI MQALQNFGLC	600	X-ATM	YFCMEKFPLDP AMWPEKRLAY TRSVATSSIV GYIVCLGDRH	1399
H-ATM	TYEHEAMWKG ALVTYDLETA IPSSTRQAGI IOALQNLGLC	2070	H-ATM	YFCMEKFPLDP AIIPEKRLAY TRSVATSSIV GYIVCLGDRH	2868
Consensus	TYEHEA.WKG ALVT..DLE.. P..TRQAGI .QALQNLGLC		Consensus	Y FCMEKFPLDP A .WPEKRLAY TR SVATSSIV G YIVCLGDRH	
X-ATM	HM1SLTYRLGL EHENAEMSS LEIHTHQAAW RNMQWDDSLP	640	X-ATM	VQNLILDEES AELVHIDLGV AFEQGKILPT PETVPPRLTR	1439
H-ATM	H1LSVYKLG DYENKDWCP ELEHHYQAAW RNMQWDDHCT	2110	H-ATM	VQNLINLDEES AELVHIDLGV AFEQGKILPT PETVPPRLTR	2908
Consensus	H..L.S.YL.GL ..EN..W..E L.B.E.QAAW RNMQWDDHCT		Consensus	V QNLILDEES A .ELVHIDLGV A FEQGKILPT P ETVPPRLTR	
X-ATM	TKNETSGPGY HESLYRAVQ S LRDKFCGFB DHIKYARVKE	680	X-ATM	DIVDGMGIGT VEGVFRRCCE KTMEMVRNQ EALLTIVEVL	1479
H-ATM	VSKEVEGSTY HESLYNALQ S LRDRPESTY ESLHYARVKE	2150	H-ATM	DIVDGMGIGT VEGVFRRCCE KTMEMVRNQ EALLTIVEVL	2948
Consensus	...B...G..Y HESLYA.QS LR.D.BF..P. ...KYARVKE		Consensus	D IVDGMGIGT V EGVFRRCCE K TMEMVRN.Q E ALLTIVEVL	
X-ATM	VEELCSGSLE SVYSLYPALC RLQAIQELAS VQGMFSRSIT	720	X-ATM	LYDPLFDWTM NPLKALYLQO --DEVDLNA TLGGDDPBCN	1516
H-ATM	VEEMCKRSLE SVYSLYPALC RLQAIQELAS IGLPFSRSIT	2190	H-ATM	LYDPLFDWTM NPLKALYLQO RPEDETELHP TLNADDPBCN	2988
Consensus	VEE..C..SLB SVYSLYP.L. RLQAIQEL.S .G..FSRS.T		Consensus	LY DPLFDWTM N PLKALYLQO ..DB..L.. TL..DD..EC.	
X-ATM	DDGLKDFFKL WORQSLQKL SDFFELPVL SLRSLVILEL	760	X-ATM	RNSCD-SQSV NKLSRLFPGW KAWVZ 1580	1555
H-ATM	HRQLSSEVYK WQKHSQKL SDFFPQEPIM ALRTVPL	2230	H-ATM	RNLSDIDQSF DKVAERVLMR LQEKLKGVVE GIVLVSVGQV	3028
Consensus	..L..K..W.Q..S.QLKLSD F.P.B..P..L.R.VILE.L		Consensus	R N..D..Q.S. .KVAERVL.R L QEKLKGVVE G .VLSVGQV	
X-ATM	LOQEKKOPSQ ESLKDILTKH LLDSLRSIAR AGNTQLPEKA	800	X-ATM	NHLLQQAMDP KNLSRLFPGW KAWVZ 1580	
H-ATM	MBKEMDNRSB ECIKDILTKH LVLSELILART FNTQLPERA	2270	H-ATM	NLLIQQAIDP KNLSRLFPGW KAWVZ 305	
Consensus	...B.....E..KDLITKH L..L..AR..NTQLPEA		Consensus	N .LIQQA.DP KNLSRLFPGW KAWVZ.	

Figure 1 (a) ATM domains and cloning strategy. The human ATM protein is represented by a thick black line, specific domains are indicated. X-ATM clones are indicated by arrows. T7/TBH-XTC denotes the *in vitro* translated fragment of X-ATM used to screen sera from rabbits A and B. PQE60/TBH6H was used in antigen production and part of this region was used in RNase protection assays (construct T7/TBH-XTC). (b) Sequence comparison of X-ATM and human ATM. Fifty per cent of the predicted sequence of *Xenopus* ATM (X-ATM) was aligned with the amino acid sequence of human ATM (H-ATM) using Geneworks software (Intelligenetics). Identical amino acids between the two proteins are indicated in bold letters below the comparison. The regions of the human sequence that were used to derive degenerate oligonucleotides are underlined (thin lines). The PI3 kinase domain consensus region is underlined (thick line). The nucleotide sequence that was used to translate X-ATM has been deposited to GenBank, accession number AF174488.

X-ATM is present in a protein complex in *Xenopus* extracts

The carboxyl-terminal kinase domain of ATM represents a small portion of this large protein. Little is known about the function of the domain(s)

outside the kinase region but support for its importance comes from both genetic analysis of ATM mutants (Concannon and Gatti, 1997) as well as the finding that a putative leucine zipper region of ATM behaved as a transdominant negative (Morgan *et al.*, 1997). A central proline rich domain has already been shown to interact with the c-abl SH3 domain (Shafman *et al.*, 1997), and the remainder of the protein is suspected of playing regulatory functions by interacting with other



Figure 2 Characterization and specificity of antibodies against X-ATM. (a) Specificity of the crude serum and the affinity purified antibody for X-ATM. One hundred µg of *Xenopus* egg extract was electrophoresed (6% SDS-PAGE) and Western blotted with the crude serum A (lane 1), the crude serum B (lane 2) or the affinity purified antibody from serum B (lane 3). In all cases a polypeptide of 370 kDa was detected. Lane 4 is a human cell extract (HeLa) probed with an anti-human ATM antibody (Gately *et al.*, 1998). (b) Immunoprecipitation of native X-ATM protein. Five hundred µg of extract were subjected to immunoprecipitation followed by Western blotting using the X-ATM antibody (lane 2). A 370 kDa polypeptide of the same size as that in the control extract (lane 1) is detected

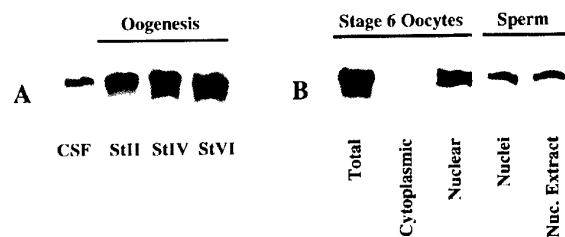


Figure 4 (a) X-ATM during oogenesis. Manually dissected oocytes at different stages of vitellogenesis were collected. Equal amounts of protein were separated by electrophoresis and subjected to Western blotting. The stages of oogenesis are indicated below the lanes. The mobility was compared with that of X-ATM in a CSF extract (CSF). (b) Subcellular localization of ATM in the germline. Protein samples from either complete stage VI oocytes (total), stage VI oocyte cytoplasm (cytoplasm) or stage VI oocyte nuclei (nuclear) were processed for Western blotting. The two right lanes show a similar Western blot using either demembranated sperm nuclei or protein extracts prepared from nuclei

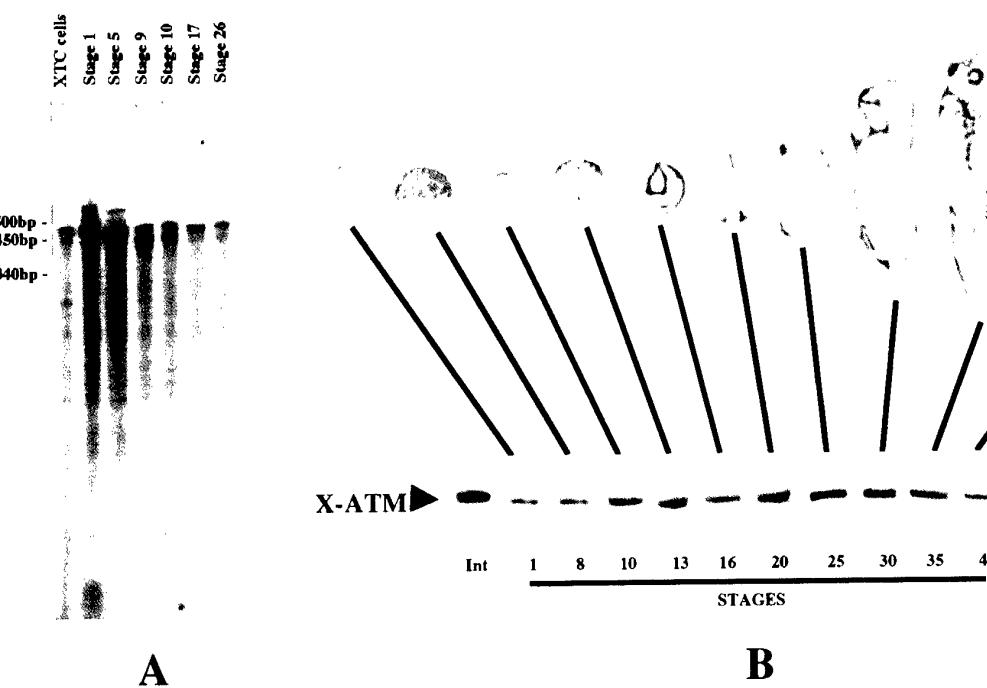


Figure 3 (a) Expression of X-ATM mRNA during development. RNase protection assays using a 480 bp probe from clone TBH4 of the X-ATM cDNA were performed using standard protocols at different stages of development (as indicated above the lanes) and from RNA prepared from the *Xenopus* XTC cell line (1st lane). The protected band is 480 bp. (b) Expression of X-ATM protein during development. Equal amounts of protein extracts prepared from different stages were separated by electrophoresis followed by Western blot with the ATM affinity purified antibody. The protein is detected from stage 1 and the level of expression increases slightly through gastrulation (St. 13), and peaks at the time of formation of the neural tube and somites (St. 15–25), to slowly decrease thereafter. Drawings of the embryonic stages are shown above the autoradiogram

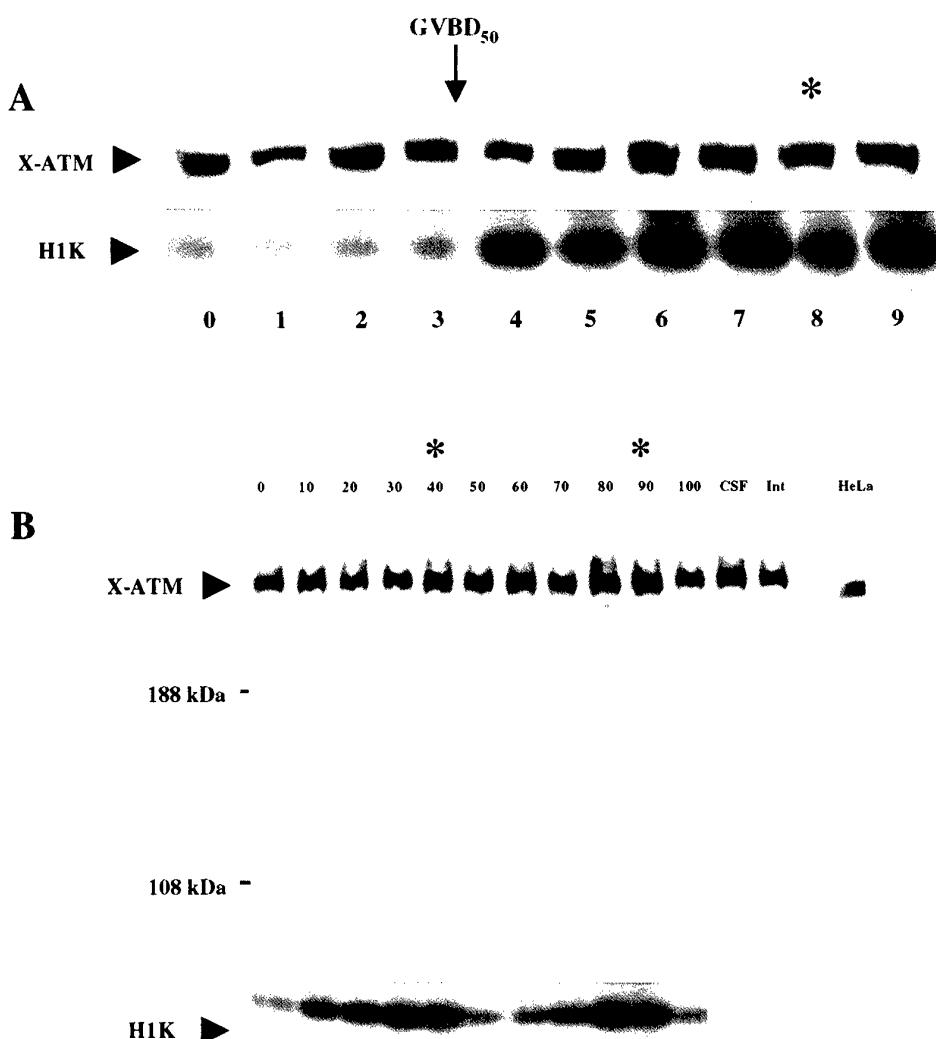


Figure 5 (a) X-ATM expression throughout the meiotic cell cycle. Western blot analysis of oocytes progressing synchronously through meiosis was performed. $t=0$ is the time of addition of progesterone. GVBD₅₀ indicates the G2/M transition, the * indicates the interphase between metaphase of meiosis I and metaphase of meiosis II. X-ATM Western blot: top panel, Histone H1 kinase activity of the same samples: bottom panel. (b) X-ATM during the mitotic cell cycles. ATM levels during S phase and mitosis were compared by Western blot analysis of 'cycling extracts' prepared from *Xenopus* eggs. X-ATM levels were monitored by 6% SDS-PAGE and Western blot analysis of extracts over a period of 100 min (top panel), corresponding to two cell cycles as determined by analysis of cdc2/cyclin B kinase activity (bottom panel). M phase, i.e. the kinase activity peaks, is indicated by *. Comparison of a mitotic extract (CSF), and an interphase extract (INT), also indicated a similar amount of ATM in these different cell cycle phases. For comparison, a HeLa whole-cell lysate was also analysed on this gel. Human ATM antibody (Gately *et al.*, 1998) recognizes a band with a slightly faster mobility than *Xenopus* ATM

proteins. To determine whether X-ATM might be associated with other proteins in *Xenopus* extracts we analysed extracts by native gel electrophoresis. We detected a native, high molecular weight X-ATM-containing complex with an estimated size of 500-kDa (Figure 7). In addition, using sucrose gradient centrifugation, we detected a high molecular weight form of ATM in extracts consistent with our electrophoresis analysis (data not shown).

Discussion

Xenopus ATM

We report the cloning of a *Xenopus* homolog of ATM, the gene mutated in Ataxia-Telangiectasia.

Although we have not yet isolated the full-length clone, sequence comparison spanning 50% of the predicted ORF, establishes clearly that we have isolated a *Xenopus* ATM homolog rather than a member of the PI3-kinase family. The amino acid levels of identity between X-ATM and human or mouse ATM are 71 and 69%, respectively while human ATM is 85% identical to mouse ATM over the same half of the protein. In comparison, there is only weak homology outside the kinase domain between X-ATM and ATR, mei41 or rad3, three genes which are forming the 'ATR/rad3' branch of this family of protein kinases (Hoekstra, 1997).

The high degree of conservation between X-ATM and ATM within the kinase domains (85% identity) strongly suggests that the catalytic activity of these two proteins will be conserved. The decreasing levels of

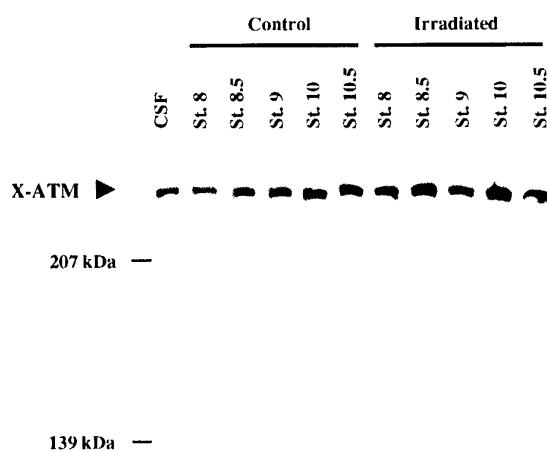


Figure 6 X-ATM protein following DNA damage. The level of ATM in extracts from control and γ -irradiated embryos between stage 8 and 10.5 was compared. Embryos were γ -irradiated (40 Gy) using a ^{60}Co source at stage 2. Embryos were collected and crushed at the indicated stages, and the soluble extract was analysed by 6% SDS-PAGE followed by Western blot analysis. ATM in a mitotic extract (CSF) is also shown

identity outside the catalytic domains could reflect potential differences in regulation of these proteins between species.

Mutations in the ATM gene are not clustered in a specific area of the molecule or restricted to a limited number of residues, instead they span the entire ORF of the molecule. This has made it difficult to discriminate between true mutations and polymorphisms. The availability of X-ATM sequence might help resolve some of these issues by identifying mutations in conserved residues that have been originally classified as polymorphisms. Conversely, it might help confirm the location of polymorphisms in non-conserved residues.

Cell cycle patterns of X-ATM expression

We present a detailed analysis of the expression of X-ATM throughout both meiotic and mitotic cell cycles. These cell cycles are naturally synchronous in *Xenopus* and allowed the cell cycle expression of ATM to be followed in undisturbed cell cycles. We find no evidence for cell cycle oscillation in the level or the electrophoretic mobility of ATM protein during the mitotic cell cycles in cell-free extracts or during the meiotic divisions of the oocyte. This confirms previous results for human ATM obtained in cells that have been synchronized in culture (Brown *et al.*, 1997; Gately *et al.*, 1998). Due to the large size of X-ATM, small variations in mobility might not be detectable despite the use of very resolute PAGE. In addition, ATM could be modified in ways that does not affect its electrophoretic mobility. Therefore we cannot rule out that X-ATM protein could be modified in a cell cycle dependent manner.

Developmental expression of X-ATM

We found that X-ATM was expressed maternally, very early during oogenesis. This might reflect the need for

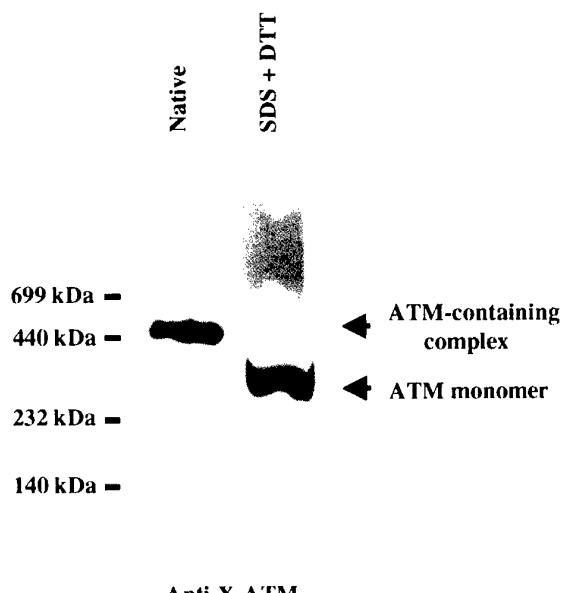


Figure 7 X-ATM complexes. Two hundred μg of egg extract were electrophoresed under native conditions and immunoblotted with X-ATM antibody (first lane). A band of approximately 450–500 kD corresponding to the complex containing X-ATM is observed as indicated. The same sample was electrophoresed in the same polyacrylamide gradient gel under reducing and denaturing conditions (second lane). The immunoreactive band migrates at 370 kDa, the molecular weight of monomeric ATM

ATM function in growing oocytes and during meiosis. Later during embryonic development we did not observe dramatic changes in the levels of mRNA or protein which is consistent with X-ATM playing a role in all cell types throughout development. RNase protection assays show the presence of a second protected fragment at stages 9 and 10 of smaller size than the expected protected fragment. This could be due to the two different mRNA species generated by alternative splicing. Alternatively, a stable secondary structure could exist at either end of the protected fragment giving rise to the observed pattern. Since alternative mRNA splicing has not been observed within the ORF of human ATM (Savitsky *et al.*, 1995) we favor the second hypothesis.

We also followed the protein expression patterns of ATM in embryos that have been irradiated by ionizing radiation. We previously reported that such an insult induces a synchronous apoptotic program in *Xenopus* embryos, manifest at the onset of gastrulation (Anderson *et al.*, 1997; Hensey and Gautier, 1997). In such embryos destined to die by apoptosis we did not observe any changes in the levels of X-ATM. Moreover, when we followed X-ATM in *Xenopus* apoptotic extracts containing active caspase (as seen by PARP cleavage), we did not observe X-ATM cleavage despite the presence of a good consensus site for caspase cleavage (towards the C-terminal-end) of the X-ATM molecule (DIVD) (data not shown).

Localization

We clearly demonstrated that X-ATM is exclusively nuclear in the germline. *Xenopus* oocyte nuclei can be



manually isolated from their surrounding cytoplasm with virtually no cross contamination. This provides unequivocal evidence for the subcellular localization of X-ATM in this system. Although X-ATM isolated from oocytes migrated with the same mobility on PAGE as X-ATM isolated from eggs (see Figure 7), we occasionally observed a second slower migrating band on Western blots from oocytes early during vitellogenesis (data not shown).

As meiotic maturation takes place and the nuclear envelope breaks down, X-ATM becomes cytoplasmic. In the unfertilized egg, corresponding to the second meiotic metaphase, the protein is cytoplasmic as seen by its presence in cell-free extracts. The nuclear localization of the protein in oocytes might reflect the fact that X-ATM could be essential for the proper completion of meiosis as is the case in mammals (Barlow *et al.*, 1998; Xu *et al.*, 1996). However, during the early cleavages of the embryos, the rapid divisions lack cell cycle checkpoints and are relatively insensitive to either DNA replication inhibitors or DNA damage (Hensey and Gautier, 1997). It will be interesting to determine whether exclusion of ATM from the nucleus correlates with the lack of cell cycle checkpoints and at what time during development X-ATM becomes nuclear again.

It is interesting to compare the expression and localization of X-ATM with that of *Xenopus* p53 since in mammalian systems p53 has been shown to be a target and a substrate for the ATM protein kinase. *Xenopus* p53 is structurally and functionally related to human p53 (Cox *et al.*, 1994; Soussi *et al.*, 1987; Wang *et al.*, 1995). Like X-ATM, p53 is present in oocytes and synthesized during early oogenesis (Tchang *et al.*, 1993). However in contrast to what we observe for X-ATM, *Xenopus* p53 is entirely cytoplasmic in the oocyte. The difference in subcellular localization between p53 and X-ATM might provide a partial explanation for the unusually high levels of p53 protein observed in *Xenopus* oocytes and embryos.

Complex formation

We also clearly demonstrate that ATM is present in at least one larger complex than the monomeric form. The non-denaturing gels we used in these experiments allowed us to clearly resolve a complex of approximately 500 kDa, which could be disrupted following SDS treatment. It is possible that other larger complexes also exist but cannot be resolved using PAGE. We confirmed these findings using sucrose gradient centrifugation of egg extracts followed by Western blotting. We found that X-ATM was found in a high molecular weight complex sedimenting around 600 kDa (data not shown).

The finding that X-ATM is associated with other partner(s) in the simple, unregulated cell cycles of *Xenopus* cell-free extracts suggest that X-ATM might always require the association with regulatory subunits to perform its functions. ATM has been shown to interact with c-abl which is also a substrate for the kinase. It was also demonstrated that ATM interacts with β -adaptin, a protein involved in clathrin-mediated endocytosis of receptors (Lim *et al.*, 1998). The identity of the *Xenopus* ATM partner(s) awaits further investigation.

Materials and methods

Cloning of *Xenopus Atm*

Degenerate RT-PCR Four degenerate primers were designed from regions of amino acid identity between the kinase domain of ATM and its yeast homologue TEL 1, with a slight bias towards the human sequence. Primers 1 and 2 were in the 5'-3' direction. Primers 3 and 4 were in the 3'-5' direction. The primer sequences were as follows:

Primer 1: 5'-GCGCGGGATCCGA(CT)GA(CT)(CT)T(AG-CT) (AC) G (AGCT) CA (AG) GA (CT) -3' (amino acids D-LRQDA, positions 2719–2726 in ATM)

Primer 2: 5'-GCGCGGGATCCT(AGCT)ATG(GC)A(AG)-CA (AG) GT (A GCT) TT (CT) -3' (amino acids MQQVFQ, positions 2727–2733 in ATM)

Primer 3: 5'-GCGCATCGATA (CT) (AGCT)CC(AGCT)-A(AG)(AG)TC(ATG)AT(AG)T G-3' (amino acids HIDLG-V, positions 2886–2892 in ATM)

Primer 4: 5'-GCGCATCGATT(AGCT)CC(CT)TG(A-GCT)TC(AG)AA(AGCT)GC-3' (amino acids AFEQGK, positions 2892–2898 in ATM).

Total RNA was isolated from *Xenopus* Tissue Culture (XTC) cells (RNAzol B, Tel-Test) and was subjected to a reverse transcription reaction. The cDNA product was used as a template in PCR with primers 1 and 4 in a standard PCR reaction. A fragment of approximately 480 bp was amplified, and reamplified with nested degenerate primers 2 and 4.

Library screens A λ Zap library from XTC cells was screened using the 480 bp PCR fragment as a probe and a high stringency hybridization was performed. Following three successive rounds of screening the clone XTC 3 was isolated containing a 2.1 kb insert including 600 bp of 3' UTR.

A further 500 bp of *X-Atm* was cloned upon screening of a second λ Zap library from Stage 25 *Xenopus* embryos (a gift from Dr Hemmati-Brivanlou and Dr Harland). The TBH 4 clone was 1 kb in size and partially overlaps with the 5' 500 bp of clone XTC 3 (see Figure 1a).

X-Atm library construction A Zap Express library was constructed using the Zap Express cDNA Gigapack III Gold Cloning kit, (Stratagene). Messenger RNA was isolated from XTC cell total RNA, and subjected to first strand synthesis using a primer specific for *X-Atm* (instead of the oligo DT primer provided) containing a *Xba*I site 5'-GAGAGAGAGACTA GTCTCGACTGTGTGACAAT-GTGACGTGC-3'. All cDNA's with molecular weights above 1 kb were excised, packaged into λ arms and the library titrated.

Subsequent screening of 20 000 plaques revealed a 2.5-kb clone, *X-Atm* 1.3 that overlaps with the 5' 210 bp of the TBH 4 clone (Figure 1a).

5' RACE mRNAs were isolated from XTC cells as previously mentioned and reverse transcribed. Second strand synthesis and adapter ligation was carried out using the Marathon 5' RACE kit (Clontech). Long distance PCR was performed on the cDNA. After three rounds of PCR with nested primers an 800 bp product, clone RACE 1, was generated and resulted in a further 500 bp of new sequence (350 bp of this clone overlaps with the 5' of clone *X-Atm* 1.3, see Figure 1a).

The assembly of all preceding clones provided 5.3 kb of sequence corresponding to the 3' UTR and 50% of the predicted ORF of the cDNA. Amino acid sequence alignments and alignment scores were performed using Geneworks software.

RNAse protection assay

The RNAse protection assay was performed according to the protocol described in the RPAII kit (Ambion). A 480-bp fragment from clone TBH 4 (nucleotide position 5–485) was used as a probe (Figure 1a). The probe was hybridized with 15 µg of XTC cell total RNA and with RNA from samples of ten *Xenopus* eggs or embryos, stages 5, 9, 10, 17 and 26. After hybridization the samples were digested with RNAse A and H, and analysed by PAGE.

Bacterial expression of X-ATM fragment and generation of X-ATM antibodies

A 500 bp fragment from *X-Atm* clone TBH 4 (positions 5–485) was amplified by the forward primer 5'-GACGTCTCT-TAATAATCTGATT-3' upstream of an internal *Nco*I site and the reverse primer 5'-GCGCGGATCCGAGAT-TAAGGCCCGGCCAG-3' which contains a *Bam*HI site (Figure 1a) and cloned into the *Nco*I and *Bam*HI sites of the His₆ Expression vector PQE60.

We also cloned the 3' 1.5 kb of the *X-Atm* ORF into a T7 vector that allowed the production of a 65-kDa polypeptide in rabbit reticulocyte lysate (T7/TBH-XTc).

X-ATM was expressed in *E. coli* and affinity purification of the denatured peptide was carried out as described in the QIAexpress protocol (Qiagen). 2 mg of protein was run on a 15% preparative polyacrylamide gel, the band excised from the gel and used to immunize rabbits.

Sera were tested by Western blotting against the *in vitro* translated X-ATM. Sera from two rabbits gave a strong signal and the antibodies from rabbit B were further purified on an affinity column prepared by coupling the 20 kDa X-ATM protein fragment to CNBr sepharose beads.

Protein sample preparation

Cycling and CSF extract Cycling extracts were prepared from unfertilized *Xenopus* eggs, as described in (Murray, 1991).

Embryos Batches of 20 embryos from the indicated stages were collected and snap frozen in liquid nitrogen. The embryos were crushed in a modified RIPA lysis buffer (150 mM NaCl, 50 mM Tris, pH 7.4, 0.5% Nonidet P-40, 0.05% SDS) containing pepstatin, leupeptin, aprotinin and AEBSF (10 µg/ml each). One hundred µg of protein from each sample used in Western blots.

Oocytes Stages II, IV, and VI (Dumont, 1972) were gently crushed in 150 mM NaCl, 50 mM Tris, pH 7.4 and processed in the same way as embryos. Oocyte maturation, i.e. meiosis, was induced *in vitro* by 10 µM progesterone in MBS-H (Gautier and Maller, 1991).

Cells XTC cells were washed in ice-cold PBS, pelleted and resuspended in RIPA lysis buffer (150 mM NaCl, 50 mM Tris, pH 7.4, 1% Nonidet P-40, 0.5% sodium deoxycholate, 0.1% SDS). Nuclear Protein Extracts (NPE) were prepared according to (Walter et al., 1998).

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Western blotting

Protein samples were separated under denaturing conditions on 20 cm, high resolution 6% SDS polyacrylamide gels, and transferred to nitrocellulose. After blocking in 5% milk in PBS, membranes were incubated with either affinity purified X-ATM antibody or human ATM antibody AT 1.8 (a gift from Dr T Yen), followed by incubation with horse-radish peroxidase-conjugated secondary antibodies. Proteins were visualized using enhanced chemiluminescence (Amersham).

Quantitation of X-ATM in Xenopus embryos

Linear T7/TBH-XTc clone (Figure 1a) was used as a template in an *in vitro* transcription/translation reaction with 2 µl of [³⁵S]methionine of known specific activity. One µl samples from the completed reaction were counted in a scintillation counter to determine the average number of counts corresponding to the calculated number of moles of [³⁵S]Met. The amount of [³⁵S]Met incorporated into the X-ATM band was then determined by electrophoresing 1–3 µl of the translation reaction on a 10% SDS-PAGE gel and transferring to nitrocellulose. The radiolabeled band was excised and counted, and counts were converted to pmoles of [³⁵S] per µl of the translation. Western blots of both the translation products and CSF extract were carried out in parallel and the band intensities of different dilutions were compared and hence, knowing the number of methionines in the translation product, we were able to determine the molarity of X-ATM in CSF extract. This was extrapolated to embryos by comparing band intensities in Western blots containing CSF and extracts from a known number of embryos resulting in an estimation of the molar concentration of X-ATM per embryo.

Native gradient gel electrophoresis

Ten µl of interphase extract were either suspended in TBE containing 10% glycerol or a modified buffer also containing 3% SDS and 100 mM DTT. Samples in the SDS/DTT containing buffer were heated to 80°C for 5 min. Proteins were then separated on a non-denaturing linear gradient polyacrylamide gel in 1×TBE overnight. Before transferring proteins to nitrocellulose the gel was soaked in SDS buffer (48 mM Tris, 39 mM glycine, 0.25% SDS) for 30 min at room temperature with agitation, followed by a 30 min soak at 80°C (Hendrickson et al., 1996). Proteins were probed with the X-ATM antibody as previously described.

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EXPRESSION NOTE

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Expression and subcellular localization of X-ATM during early *Xenopus* development

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Abstract ATM, the gene mutated in ataxia telangiectasia, is a protein essential for handling DNA strand breaks. We recently isolated the *Xenopus* homologue of ATM, X-ATM and we report here the detailed expression pattern of the protein and the mRNA during early *Xenopus* development. During the cleavage stages, ATM protein was concentrated in and around the nuclei of all cells with low levels of expression also detected in the cytoplasm. Following neurulation, increased protein levels were detected in the nuclei of developing somites and in the central nervous system. Areas of high protein expression correlated with areas of increased mRNA expression which was detected in the nuclei of somites and the developing lens.

Key words Ataxia Telangiectasia · ATM · *Xenopus* · Lens · Somites

The disease ataxia telangiectasia results from mutations of the ATM gene (Savitsky et al. 1995) and is characterized by a variety of clinical manifestations, including neurodegeneration, immunodeficiency, and a predisposition to cancer. At the molecular level the ATM protein regulates cellular responses to DNA strand breaks (Banin et al. 1998; Brown et al. 1997; Canman and Lim 1998). Previous studies have shown that Atm is localized to meiotic nuclei (Barlow et al. 1998) and the developing

nervous system in mice (Herzog et al. 1998). ATM mRNA is ubiquitously expressed in mouse embryos with elevated mRNA expression levels in the cerebellum and other regions of the CNS suggesting an early developmental requirement for ATM in the nervous system (Chen and Lee 1996; Soares et al. 1998). However, little is known about ATM expression during early development. We cloned the *Xenopus* homologue of human ATM (Robertson et al. 1999), and using whole-mount in situ hybridization and immunohistochemistry, have analyzed the expression pattern of X-ATM during early development in *Xenopus* embryos.

Using an antibody that specifically recognizes XATM in immunoblotting and immunoprecipitation experiments (Robertson et al. 1999), we analyzed Atm expression by whole-mount immunohistochemistry at representative stages of *Xenopus* development (Nieuwkoop and Faber 1956). Atm is expressed in all blastoderm nuclei in a stage 8 embryo (Fig. 1 A). Sectioning embryos labeled in whole-mount revealed that Atm is predominantly nuclear and perinuclear as evidenced by the overlapping DAPI stain (Fig. 1B, C). However the staining is not homogeneous throughout all nuclei. A component of the protein is cytoplasmic and is also detected in a fiber-like network radiating from the dense area of nuclear staining through the cytoplasm (Fig. 1D). Punctate areas of stronger staining are detected along the fibers. Similar expression patterns were detected in 32-cell (stage 6) and 64-cell (stage 7) embryos and we did not observe any change in the expression pattern following γ -irradiation (data not shown). These observations are consistent with our previous observation that Atm is nuclear in both the male and female germline in *Xenopus* (Robertson et al. 1999). A number of biochemical studies have also shown that human Atm is a nuclear protein (Brown et al. 1997; Chen and Lee 1996; Gately et al. 1998).

The protein was ubiquitously expressed during gastrulation and early neurulation with the highest level of staining detected in the nuclei (data not shown). By mid to late neurulation (stage 18), differential tissue expres-

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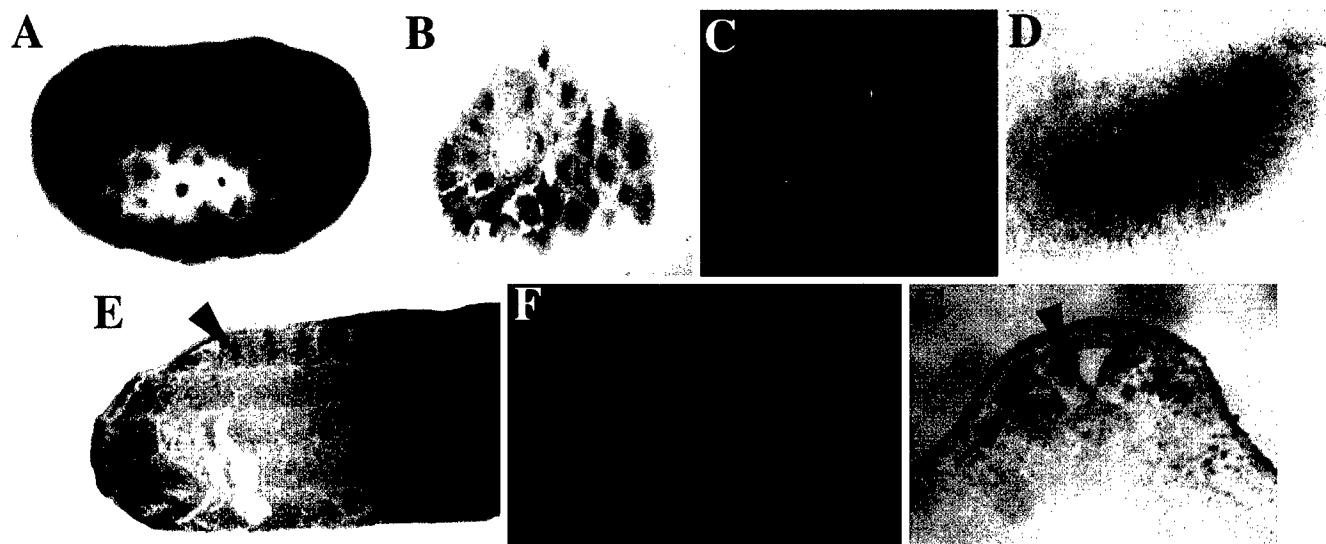


Fig. 1A–G Atm protein expression and subcellular localization in stage 8 and stage 21 embryos. Immunohistochemistry was performed using a 1:1000 dilution of affinity purified α -ATM (Robertson et al. 1999). **A** Stage 8, lateral view, whole-mount α -Atm showing strong nuclear staining. Embryos processed in a similar manner but omitting the primary antibody showed no staining. **B** Section from a whole-mount embryo. **C** The same section DAPI-stained, showing nuclear staining overlaps with ATM stain-

ing. In some cells the strong Atm staining masks the DAPI stain. **D** Higher magnification of a section showing Atm staining in a single cell. **E** Stage 21, parasagittal section. The strong somite staining is due to high levels of Atm expression in the somite nuclei (arrowhead). **F** DAPI staining of the same section as in **E**, indicating the localization of the nuclei. **G** Stage 21, transverse section. High levels of Atm expression in both the nuclei of the somites (arrow) and the neural tube (arrowhead) are observed

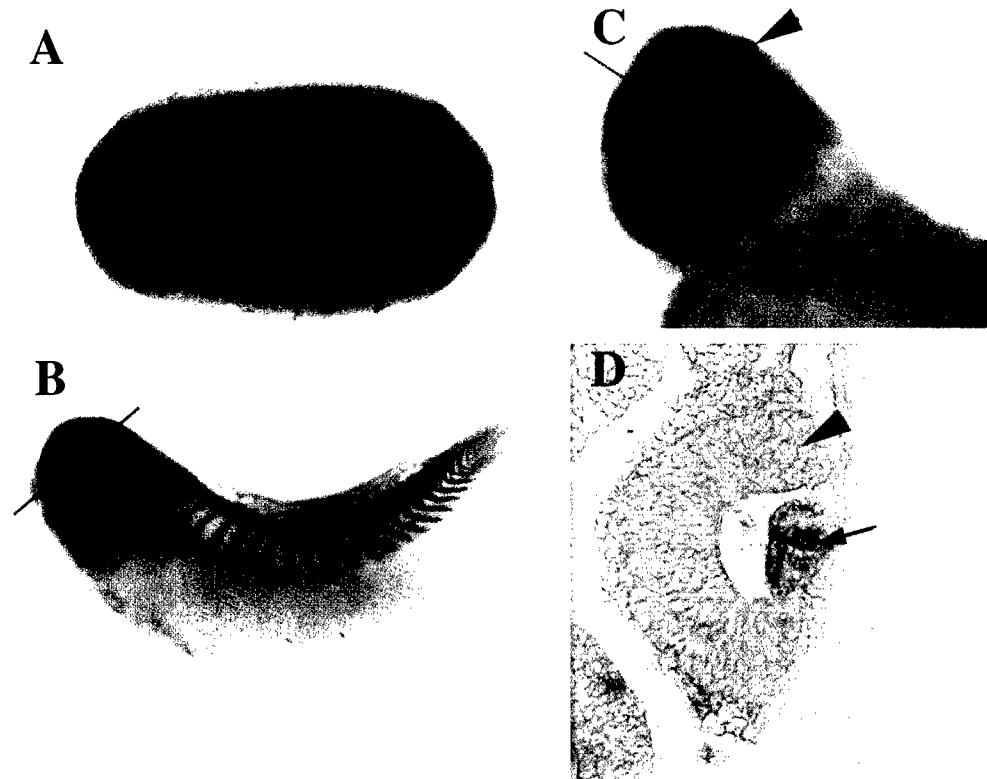


Fig. 2A–D Atm mRNA expression at stage 18 (**A**), and 32 (**B–D**). A 2.5 kb fragment of the *X-ATM* cDNA, corresponding to *X-ATM* 1.3, a region 5' of the putative kinase domain (Robertson et al. 1999), was transcribed to generate a digoxigenin labeled antisense probe. Atm mRNA expression is detected in the somites and developing CNS (anterior staining) at stage 18 (**A**). **B** Stage 32, lateral view, line indicates the section shown in **D**. High levels of mRNA are maintained in the somite nuclei and are also detected in the branchial arches, developing brain and eye. **C** Stage 32, a

shorter staining time reveals more detail in the anterior staining. The lens nuclei are strongly stained (arrow) and specific regions of the brain show high levels of mRNA expression (arrowhead). **D** A transverse section at the level of the mesencephalon reveals a high level of mRNA expression in the developing lens (arrow) with a lower level of expression throughout the retina (arrowhead). Embryos processed in a similar manner but omitting the antisense probe showed no staining

sion, as evidenced by increased protein levels in the central nervous system and somites, became apparent (data not shown). As seen in Fig. 1 E and G, the nuclei of the developing somites and neural tube were most strongly stained at stage 21. Some staining of the epidermis was also observed. The protein continued to be expressed throughout the developing embryo, and by stage 31/32, elevated expression levels were maintained in the somite nuclei in addition to the developing CNS and the retina and lens of the developing eye (data not shown).

High background staining prevented the detection of specific mRNA expression patterns prior to stage 17, but beyond this stage, increased levels of mRNA expression correlated with areas of increased protein expression. Starting at mid to late neurulation, mRNA expression was detected in the developing somites (Fig. 2A, B). In the stage 18 embryo shown in Fig. 2A, mRNA is highly expressed in the somite nuclei and this high level of expression is maintained up to stage 37/38, the period of somite differentiation. In addition to high levels of expression in the somite nuclei, a stage 32 embryo also shows high mRNA expression in the branchial arches, brain, and developing eye, particularly the lens (Fig. 2C, D).

The predominantly nuclear localization of Atm is consistent with its putative role in regulating the cellular response to DNA strand breaks. The ubiquitous expression pattern is also consistent with its function in the DNA damage response, an important cellular response. Studies in mouse have also shown increased expression of ATM in the central nervous system, pointing to an early developmental requirement for ATM in the nervous system (Chen and Lee 1996; Soares et al. 1998). The localized expression such as that detected in the developing somites and lens might reflect a physiological function involving DNA breaks. ATM, among other genes, is believed to be involved in DNA damage recognition (Smith et al. 1999). This role is critical in cycling cells as a component of the checkpoint pathway, but could also play a role during development and differentiation. Interestingly, mutations in several genes implicated in DNA end-joining and ligation show developmental abnormalities (Barnes et al. 1998; Tebbs et al. 1999).

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Reconstitution of an ATM-Dependent Checkpoint that Inhibits Chromosomal DNA Replication following DNA Damage

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Summary

Cell cycle checkpoints lead to the inhibition of cell cycle progression following DNA damage. A cell-free system derived from *Xenopus* eggs has been established that reconstitutes the checkpoint pathway inhibiting DNA replication initiation. DNA containing double-strand breaks inhibits replication initiation in a dose-dependent manner. Upon checkpoint activation, a prereplicative complex is assembled that contains ORC, Cdc6, Cdc7, and MCM proteins but lacks Cdc45. The checkpoint is ATM dependent. Cdk2/CyclinE acts downstream of ATM and is downregulated by Cdk2 phosphorylation on tyrosine 15. Cdk2AF/CyclinE is refractory to checkpoint signaling, and Cdc25A overrides the checkpoint and restores DNA replication. This report provides the description of a DNA damage checkpoint pathway that prevents the onset of S phase independently of the transcriptional function of p53 in a vertebrate organism.

Introduction

Cells respond to DNA damage by activating checkpoint pathways that delay progression through the cell cycle (Hensey and Gautier, 1995; Elledge, 1996). These signaling pathways operate throughout the cell cycle. Activation of a DNA damage checkpoint can induce G1, S, or G2 phase delay. These surveillance mechanisms are essential to maintain the integrity of the genome. They ensure that damaged DNA templates are neither replicated nor segregated to the daughter cells until repaired. Failure to monitor and to signal following DNA damage is a hallmark of cancer cells (Hartwell and Kastan, 1994).

A regulatory network of proteins has been identified that participates in DNA damage checkpoint signaling. Central to this network is the ATM protein, the product of the gene mutated in the human disease ataxia-telangiectasia (Savitsky et al., 1995). Ataxia-telangiectasia (A-T) is an autosomal recessive disease that displays a complex phenotype (Boder and Sedgwick, 1970; Shiloh, 1998). Patients exhibit a progressive cerebellar ataxia, in addition to severe immune deficiencies, gonadal atrophy, telangiectases, increased risk for cancer—particularly lymphomas—and radiation sensitivity. Cell lines from A-T patients show enhanced radiosensitivity to ionizing radiation (Lavin and Shiloh, 1997), increased chromosomal loss and breakage, and abnormal telomere morphology (Smilenov et al., 1997; Vaziri et al., 1997). A-T cells are defective in cell cycle checkpoints in G1, S, and G2 (Meyn, 1995; Beamish et al., 1996; Hoekstra, 1997). The cellular phenotype of A-T suggests a defect in handling DNA breaks formed by external insults or as a result of normal physiological processes such as meiotic recombination and maturation of the immune system. ATM is a serine/threonine kinase for which structurally and functionally similar proteins have been identified and characterized in *S. cerevisiae*, *S. pombe*, *D. melanogaster*, and *X. laevis* (Weinert, 1992; Hari et al., 1995; Bentley et al., 1996; Robertson et al., 1999; Sibon et al., 1999). When exposed to ionizing radiation, mammalian ATM^{−/−} cells cannot prevent S phase entry and undergo radio-resistant DNA synthesis (RDS) (Jeggo et al., 1998).

Following ionizing radiation, ATM phosphorylates and participates in the activation of p53 (Banin et al., 1998; Canman et al., 1998; Khanna et al., 1998). Activated p53 promotes the synthesis of p21, a cyclin-dependent kinase inhibitor with preferential affinity for Cdk2/CyclinE, delaying cell cycle prior to S phase entry. ATM also phosphorylates and activates the Chk1 and Chk2 protein kinases (Matsuoka et al., 1998; Chaturvedi et al., 1999; Chen et al., 1999). The activation of Chk1 is essential to prevent entry into mitosis in mammalian cells and in *Xenopus* extracts following DNA replication block (Kumagai et al., 1998a). Chk1 mediates G2 arrest by phosphorylating Cdc25 tyrosine phosphatase at a serine residue, creating a binding site for 14-3-3, thus inhibiting Cdc25 activity (Furnari et al., 1997; Sanchez et al., 1997; Zeng et al., 1998). Chk2, which is the vertebrate homolog of *S. pombe* Cds1 and *S. cerevisiae* Rad53, regulates p53 directly by phosphorylation (Chehab et al., 2000; Hirao et al., 2000; Shieh et al., 2000). In yeast, where p53 is not present, the ATM homologs Mec1/Tel1 for *S. cerevisiae* and Rad3 for *S. pombe* function through Chk1 to regulate cell cycle arrest in G2 and are also important for G1 and S phase delay (Weinert, 1992; Siede et al., 1993; Bentley et al., 1996). In all organisms, the mechanisms by which S phase entry is prevented in an ATM/Mec1/Rad3-dependent fashion are poorly understood.

In vertebrates, the molecular dissection of the G1 arrest mechanism has mainly focused on studies of p53-dependent pathways. Responses mediated by p53 require transcription and de novo protein synthesis and therefore are more appropriate for a nonacute response to DNA damage. Furthermore, in many systems DNA

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damage induces p53-dependent apoptosis rather than cell cycle delay (Carr, 2000). It is therefore not surprising that p53-independent checkpoint signaling following DNA damage operates in mammalian cells at the onset of and throughout S phase (Lee et al., 1997; Larner et al., 1999; Agami and Bernards, 2000; Mailand et al., 2000).

Little is known about the molecular step(s) that must be blocked to prevent replication initiation in the presence of DNA damage. The initiation of DNA replication in eukaryotes requires the assembly of a prereplicative complex (pre-RC) on chromatin. The pre-RC is formed by the stepwise assembly of the origin recognition complex (ORC), the loading factor Cdc6, the putative replicative helicase mini chromosome maintenance proteins (MCMs), and Cdc45 (Stillman, 1996; Newlon, 1997), which subsequently allows polymerase loading (Mimura and Takisawa, 1998). Initiation also requires the concerted activity of the cell cycle-regulated protein kinases Cdk2/CyclinE, PKA, and Cdc7/Dbf4 (Strausfeld et al., 1994; Jackson et al., 1995; Yan and Newport, 1995; Costanzo et al., 1999; Roberts et al., 1999; Jares and Blow, 2000).

Cell-free systems derived from *Xenopus* eggs have been widely used to elucidate the biochemical bases of cell cycle transitions. They have been especially powerful in probing the regulation of entry into S phase and into mitosis. In such systems, G2 cell cycle checkpoint activity is monitored by the inhibition of nuclear envelope breakdown following experimental interference with DNA replication (Dasso and Newport, 1990; Kumagai et al., 1998a, 1998b; Guo and Dunphy, 2000).

We have designed a cell-free system to study the DNA damage checkpoint that prevents S phase entry. We monitored the effect of small damaged DNA templates on chromosomal DNA replication. This in vitro system reconstitutes a functional cell cycle checkpoint in which DNA containing double-strand breaks (DSBs) inhibits DNA replication in a dose-dependent manner. This system has revealed a novel checkpoint signaling pathway that operates through ATM. Cdk2/CyclinE is inhibited by phosphorylation of tyrosine 15 on the Cdk2 subunit, thus preventing the loading of Cdc45 at the origin of replication and initiation of DNA synthesis. This signaling is rapid, independent of de novo protein synthesis and, by inference, independent of p53 transcriptional activity.

Results

DNA Damage Checkpoint Inhibition of S Phase Entry in a Cell-Free System

To recapitulate cell cycle checkpoint response to DNA damage at the onset of S phase, we modified a cell-free system designed to study initiation of DNA replication (Chong et al., 1997). In this system, interphase *Xenopus* egg extracts are subjected to ultracentrifugation followed by sequential PEG precipitation (Figure 1 and Experimental Procedures). Two fractions (M and B) are obtained. Both fractions are required to initiate replication of chromosomal DNA assembled in an initiation-incompetent extract prepared in the presence of 6-dimethylaminopurine (6-DMAP) (Figure 2A). M contains MCM proteins, the putative replicative DNA helicase, whereas B is required for the loading of MCMs onto chromatin and for activation of the pre-RC (Chong et al., 1995).

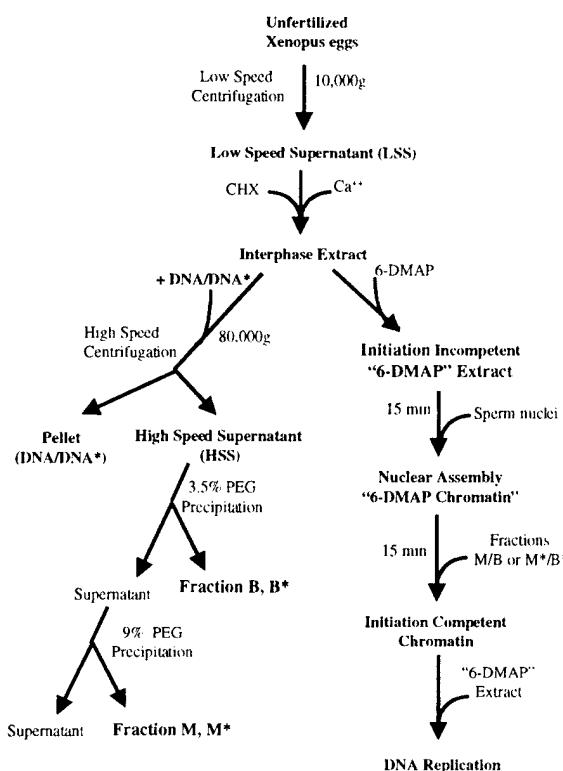


Figure 1. Fractionation Scheme and Reconstitution of an In Vitro System for the Study of the DNA Damage Checkpoint

Unfertilized *Xenopus* eggs were crushed at $10,000 \times g$. The resulting CSF-arrested low-speed supernatant was treated with cycloheximide (CHX) and calcium to promote exit from mitosis. This interphase extract was incubated with circular (DNA) or DSB-containing (DNA*) DNA molecules. Extracts were clarified by high-speed centrifugation. The resulting pellet containing all DNA molecules was discarded. The supernatant was precipitated with 3.5% PEG yielding fractions B and B*. The resulting supernatant was further precipitated with 9% PEG, yielding fractions M and M*. Initiation-incompetent extract ("6-DMAP" Extract) was obtained by treating a CSF-arrested extract with the kinase inhibitor 6-DMAP for 15 min and with calcium for an additional 15 min. 6-DMAP chromatin was prepared by isolating sperm nuclei assembled in 6-DMAP extract for 15 min to permit efficient unpacking of chromatin. DNA replication reactions were assembled by mixing fraction M and B or M* and B* in different combinations with 6-DMAP chromatin for 15 min, and replication was monitored in 6-DMAP extracts.

To activate the DNA damage checkpoint, the extract was incubated with DNA molecules containing DSBs. Purification of the treated extract yields two fractions corresponding to M and B, designated M* and B*, which were tested for their ability to support replication initiation.

DNA containing DSB was obtained by digesting double-strand circular plasmid with various restriction enzymes to yield blunt-ended fragments or fragments with 3' or 5' overhangs (Figure 2B and data not shown). Alternatively, bacteriophage λ DNA was digested with restriction enzymes to generate increasing numbers of DNA fragments (Figure 2C). Both broken and intact DNA molecules sedimented during the first ultracentrifugation step of the fractionation procedure (Figure 1) and were discarded. The M* and B* fractions were essentially free of DNA as determined by colorimetric assay and by gel electrophoresis (data not shown).

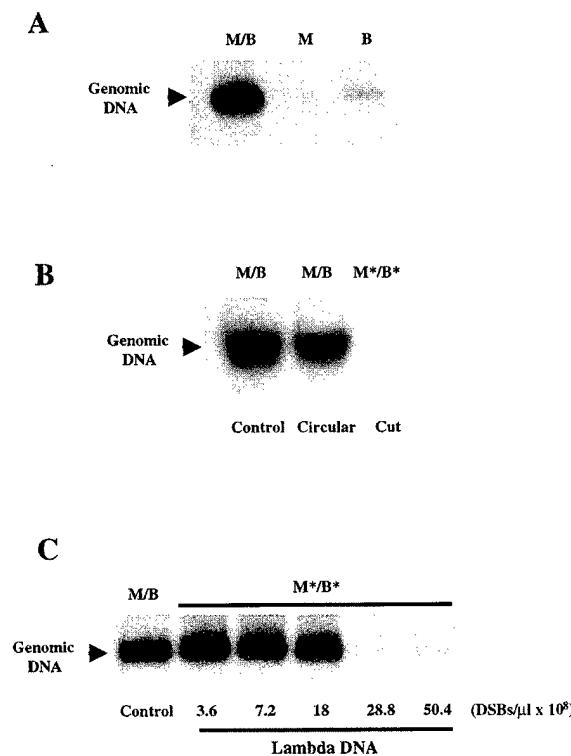


Figure 2. Inhibition of Chromosomal DNA Replication by Small DSB-Containing DNA Templates In Vitro

Genomic DNA replication was monitored by incorporation of $\alpha^{32}\text{P}$ -ATP into 6-DMAP chromatin supplemented with M and B fractions. (A) Control experiment showing replication in presence of both M and B fractions (M/B), M alone (M), or B alone (B). (B) DNA replication in presence of fractions purified from extracts incubated with DNA. No DNA: Control (M/B); uncut plasmid: Circular (M/B); plasmid digested with HaeIII containing DSBs: Cut (M*/B*). (C) DNA replication in presence of fractions purified from extracts containing an increasing amount of DSBs, as indicated.

To assay replication initiation, fractions M and B were incubated with chromatin isolated from an extract treated with 6-DMAP to permit the efficient unpacking of the highly condensed sperm chromatin. After incubation, the mixture was supplemented with initiation-incompetent extract to permit replication elongation. DNA replication was monitored by incorporation of $\alpha^{32}\text{P}$ -dATP into chromosomal DNA followed by gel electrophoresis. As previously described, fractions M and B together (M/B) supported initiation, whereas B or M alone did not (Figure 2A). In contrast, fractions M* and B* (M*/B*), obtained from extracts incubated for 15 min with fragmented plasmid DNA, were unable to promote DNA replication regardless of the type of ends (blunt, 3' or 5' overhangs) present in the damaged template. Treatment of extracts with intact circular DNA yielded M and B fractions with full activity (Figure 2B). Similarly, addition of DSB-containing DNA to extracts in the absence of incubation did not inhibit subsequent DNA synthesis (data not shown).

In an experiment performed with λ DNA digested to yield increasing numbers of DSBs, DNA replication was abolished in a dose-dependent manner above a threshold of $18-28 \text{ } 10^8$ ends/ μl (Figure 2C).

To determine which fraction was inactivated by fragmented DNA, we assembled the initiation reaction using M* and B (M*/B) or M and B* (M/B*). Figure 3A shows that M*/B supported DNA replication, but M/B* did not. This indicates that fraction B* is inactive in the initiation assay. The inactivity of B* might reflect the presence of an inhibitory signal. Alternatively, the fragmented DNA might titrate a factor(s) required for replication initiation. To distinguish between these possibilities, we mixed B, B*, and M fractions (M/B/B*) and monitored DNA replication. The presence of B* inhibited DNA replication more than 90% compared to a control reaction (M/B). We conclude that B* contains a dominant inhibitory signal (Figure 3B). The failure of B to complement rules out the possibility that B* is depleted for an essential factor.

X-ATM Is Required for S Phase Entry Checkpoint Signaling

We investigated whether the ATM protein kinase played a role in this checkpoint pathway. The 370 kDa *Xenopus* ATM (X-ATM) protein is expressed maternally and present in egg extracts (Robertson et al., 1999). We first traced the partitioning of X-ATM during the fractionation procedure using specific anti-X-ATM antibodies (Experimental Procedures). We found that X-ATM was present exclusively in the B fraction (Figure 3C). Interestingly, when the interphase extract was incubated with fragmented DNA prior to fractionation, some X-ATM cosedimented with the DNA during ultracentrifugation. In contrast, no X-ATM was present in the pellet obtained from an extract treated with circular plasmid (data not shown). This result strongly suggests that the checkpoint involves X-ATM bound to DNA (Figure 3C). We next used chemical inhibitors of ATM and ATM-like protein kinases to assess the role of X-ATM in the checkpoint response. Interphase extracts were incubated with fragmented DNA in the presence or absence of 5 mM caffeine or 200 nM wortmannin, two known inhibitors of ATM at these concentrations (Blasina et al., 1999; Sarkaria et al., 1999; Chan et al., 2000; Zhou et al., 2000). As seen in Figure 4A, fractions treated with inhibitors of ATM were able to support initiation of DNA replication to the same extent as controls. To demonstrate unambiguously the involvement of X-ATM, we fractionated an interphase extract that was preincubated with affinity-purified anti-X-ATM antibodies and fragmented DNA. The resulting M* and B* fractions were fully active in supporting replication initiation. We conclude that signaling induced by damaged DNA depends on X-ATM (Figure 4B).

Checkpoint Activation Inhibits Cdc45 Binding to Pre-RC

Initiation of DNA replication requires the stepwise assembly of protein complexes into pre-RC. We asked whether the checkpoint acted at one of the pre-RC assembly steps. Chromatin on which pre-RC components were assembled during incubation with M/B or M*/B* fractions was purified. The composition of this chromatin was determined by Western blotting with specific antibodies against different components of pre-RC. The binding of *Xenopus* ORC2, *Xenopus* Cdc6, *Xenopus* Cdc7, and all *Xenopus* MCM proteins to chromatin was unaffected by checkpoint activation (Figure 5). In contrast, Cdc45 was present only in chromatin assembled in M and B fractions (Figure 5). The failure of Cdc45 to

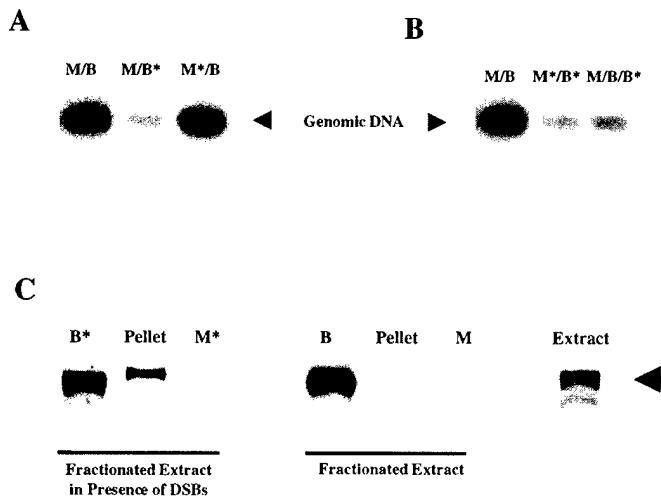


Figure 3. Fraction B* Contains X-ATM and a Dominant Signal that Inhibits DNA Replication

(A) Genomic DNA replication was monitored in extracts containing the following combinations: M/B, M/B*, and M*/B.

(B) M/B, M*/B*, and M/B/B*.

(C) X-ATM protein was traced by Western blot analysis throughout the extract fractionation procedure either in the presence of DSB-containing DNA (first three lanes) or not (next three lanes). The right lane shows an interphase extract as a control for electrophoretic mobility.

bind chromatin was X-ATM dependent, since M* and B* fractions prepared from extracts treated with anti-X-ATM antibodies supported the assembly of Cdc45 into pre-RC (Figure 5). Figure 5 also shows that the concentration of Cdc45 was identical in the M/B and M*/B* fractions (bottom panel). We therefore conclude that checkpoint activation inhibits Cdc45 assembly into pre-RC in an X-ATM-dependent fashion. Note that Cdc45 addition to pre-RC is required for loading of DNA polymerases and represents a late step in the activation of pre-RC.

Checkpoint Activation Inhibits Cdk2 Protein Kinase
 The assembly of Cdc45 into pre-RC is thought to depend upon Cdk2 activity (Mimura and Takisawa, 1998; Zou and Stillman, 1998). The influence of fragmented DNA on Cdk2 kinase was assayed in the various fractions described in Figures 3 and 4 (see Experimental Procedures). The activity of Cdk2 was significantly reduced in M*/B* compared to M/B fractions (Figure 6A). The patterns of Cdk2 downregulation and DNA replication inhibition coincided precisely. Fraction B* contained a dominant inhibitor of Cdk2 activity (Figure 6A). Furthermore, Cdk2 activity was restored to control levels in B*/M* prepared from extracts treated with caffeine, wortmannin, or anti-X-ATM antibodies (Figure 6A).

Cdk2 Activity Is Inhibited by Tyrosine 15 Phosphorylation

Cyclin-dependent kinase activity is regulated by a variety of mechanisms, including association with cyclin, phosphorylation of the catalytic subunit, and binding of inhibitory proteins. We determined the mechanism by which Cdk2 was downregulated following checkpoint activation. Cdk2 is found associated with Cyclin E in interphase *Xenopus* egg extracts (Rempel et al., 1995). The total levels of both Cdk2 and Cyclin E proteins in the various fractions were unaffected by checkpoint activation (Figure 6B). In addition, the amount of Cyclin E associated with Cdk2 was equivalent in M/B and M*/B* extracts (Figure 6B, third panel). *Xenopus* extracts contain Xic1, a maternally inherited Cdk inhibitor of the p21/p27 family (Su et al., 1995; Shou and Dunphy, 1996). We measured the association of Xic1 with Cdk2 by immunoprecipitating Cdk2 and performing a Western blot analysis with anti-Xic1 antibodies. As expected for a

member of the p21/p27 family of inhibitors, a fraction of Xic1 coprecipitated with Cdk2/CyclinE. However, this fraction was similar in M/B and M*/B*. Thus, the checkpoint does not inhibit Cdk2 by enhancing Xic1 binding (Figure 6B, fourth panel). Finally, Cdk2 downregulation correlates with phosphorylation of tyrosine 15. Using an antibody that recognizes specifically the tyrosine 15-phosphorylated epitope, we determined that Cdk2 in M*/B* was more phosphorylated than the Cdk2 in control extracts (Figure 6B, bottom panel).

We determined whether the inhibition of Cdk2/CyclinE activity and of DNA replication was due to tyrosine 15 phosphorylation. We reasoned that if tyrosine 15 phosphorylation was the major mechanism by which Cdk2 kinase activity was inhibited, a nonphosphorylatable form of Cdk2 at the inhibitory sites (Cdk2AF) should be refractory to checkpoint signaling. We added recombinant Cdk2WT/CyclinE (wild-type Cdk2) or Cdk2AF/CyclinE (a nonphosphorylatable Cdk2) to M*/B* frac-

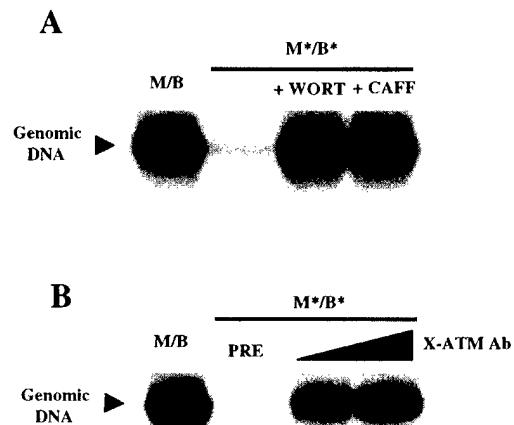


Figure 4. Inhibition of DNA Replication by DSBs Is Sensitive to Wortmannin and Caffeine and Is Dependent on X-ATM

DNA replication was monitored using fractions derived from control extract (M/B) or extracts treated with DSB-containing DNA (M*/B*). (A) Fractions M*/B* were prepared from extracts containing no drug (lane 2), 200 nM wortmannin (lane 3), or 5 mM caffeine (lane 4).

(B) Fractions M*/B* were prepared from extracts incubated with preimmune serum (lane 2) or increasing amounts of affinity-purified antibodies against X-ATM (lanes 3 and 4).

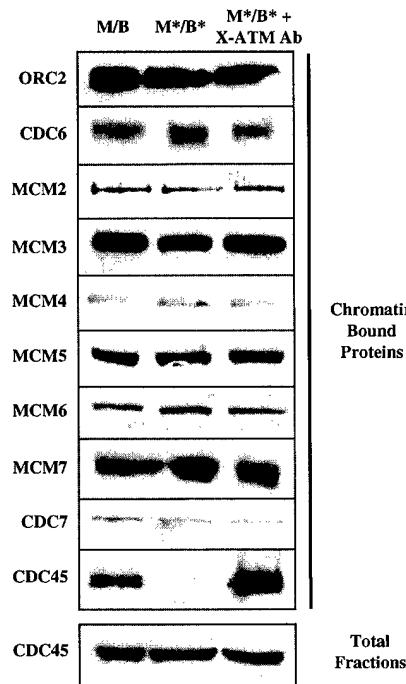


Figure 5. Consequences of Activation of the DNA Damage Checkpoint on the Pre-RC Assembly

Chromosomal DNA was purified following incubation with fractions M/B (left lane), M*/B* (center lane), or M*/B* derived from an extract incubated with anti-X-ATM antibodies (right lane). The chromatin was subjected to PAGE followed by Western blotting with specific antibodies against the pre-RC components as indicated on the left of the panels. The levels of Cdc45 protein in whole extract are shown in the bottom panel.

tions. We added identical amounts of Cdk2 kinase activity (WT or AF) corresponding to twice the endogenous Cdk2 kinase level. In these conditions, Cdk2AF/CyclinE was refractory to checkpoint signaling and restored DNA replication to M/B levels, while Cdk2WT/CyclinE did not restore DNA replication (Figure 6C). When added in a 10-fold excess over the endogenous levels, recombinant Cdk2WT/CyclinE was able to overcome the inhibition due to checkpoint signaling and restored DNA replication (data not shown). In these conditions, the replication competence of the reconstituted extract correlated with the binding of Cdc45 to chromatin (Figure 6E).

Next we measured the ability of Cdc25 to reverse the inhibitory effect of the checkpoint and restore DNA replication. We added recombinant *Xenopus* Cdc25A/WT (wild type), Cdc25A/C432A (a catalytically inactive Cdc25A), or Cdc25C/WT to M*/B* fractions (Izumi et al., 1992; Kim et al., 1999). Wild-type Cdc25A, but neither inactive Cdc25A/C432A nor Cdc25C/WT, was able to abrogate the checkpoint and restore DNA replication (Figure 6D). The end point of the DNA damage checkpoint pathway thus appears to be the phosphorylation and inactivation of Cdk2 kinase.

Discussion

Reconstitution of a DNA Damage Checkpoint In Vitro
Initiation and elongation of DNA replication are complex, multistep processes that require the assembly and the activity of a large number of proteins. In the *Xenopus*

egg cell-free system described above, DNA damage activates a checkpoint that prevents S phase entry. Although titration of replication factor(s) by fragmented DNA has been reported in other systems (Wang et al., 1999), we find instead that a dominant factor inhibits DNA replication initiation in our extracts. First, the fragmented DNA that elicits checkpoint signaling in the "sensing" extract is precipitated by ultracentrifugation and is absent from the replication extract. Second, the replication extract contains all the proteins required for replication and has not been exposed to fragmented DNA. Third, the B* fraction that contains the signaling component is dominant to B; a reaction mixture containing B/B*/M fails to support replication. Fourth, signal generation requires incubation of the extract with fragmented DNA. Fifth, the inhibitory factor is absent in B* prepared in the presence of caffeine, wortmannin, or X-ATM antibodies. These observations argue against titration of an essential factor by fragmented DNA. Instead, our cell-free system represents a true ATM-dependent checkpoint pathway and reports the reconstitution of a DNA damage checkpoint in vitro.

Interestingly, the continuous presence of damaged DNA is not required for sustaining checkpoint signaling. This supports the idea that the dominant modification induced in B* by DNA damage is stable throughout the fractionation procedure and replication assay.

It is not known whether *Xenopus* early embryos undergo S phase delay in vivo in response to DNA damage. *Xenopus* embryos undergo timely cytokinesis and cortical contraction waves following γ irradiation (Hensey and Gautier, 1997). However, DNA replication was not monitored in these experiments and, since inhibition of DNA replication does not prevent mitosis and cytokinesis in *Xenopus* (Kimelman et al., 1987), it is possible that a DNA damage checkpoint operates at the onset of S phase in these embryos.

The Checkpoint Signal

To induce the checkpoint signal, we used small double-stranded DNA templates generated by restriction endonuclease digestion of either plasmid or λ DNA. At a constant mass of 10 ng/ μ l, the checkpoint was activated at a threshold of 18–28 10⁸ ends/ μ l. In budding yeast, a single DSB generated in an artificial chromosome can initiate the DNA damage checkpoint response. It is proposed that the checkpoint signal in yeast is triggered by single-stranded DNA generated by exonuclease degradation at the site of the DSB (Lee et al., 1998). We did not detect changes in the concentration of DSB-containing DNA following signaling in the extract as measured by colorimetric assay (data not shown). Using gel electrophoresis, we did not observe detectable changes in the sizes of the DNA fragments following signaling (data not shown). Therefore, we believe that activation of the checkpoint pathway does not involve extensive processing of the DSBs. However, we cannot rule out that small modifications, such as limited processing of the ends, are taking place. Nonetheless, our results establish that DSBs are the signal that elicits the checkpoint response rather than other types of DNA lesions or free radical molecules produced following exposure of cells to ionizing radiation. Our observation that a fraction of the X-ATM cosediments with fragmented but not with intact DNA is consistent with the idea that ATM bound to DNA ends may induce the checkpoint pathway. Purified ATM has been shown to

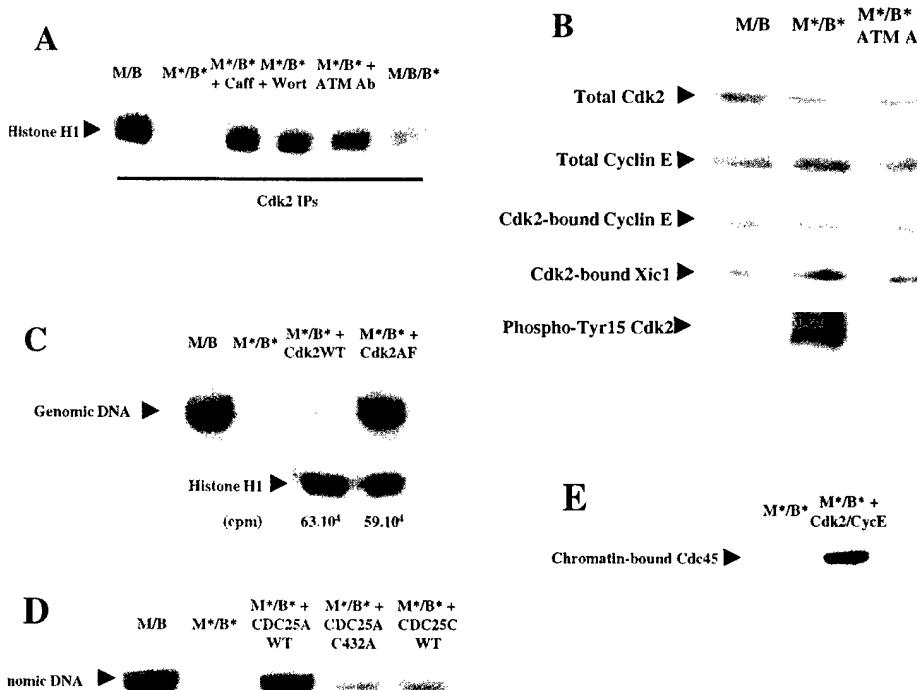


Figure 6. Activation of the Checkpoint Leads to the Downregulation of Cdk2/CyclinE and Phosphorylation of Cdk2 on Tyrosine 15

(A) Cdk2 protein kinase activity was measured using histone H1 as substrate following immunoprecipitation with an antibody specific for *Xenopus* Cdk2. As indicated, the following combinations of fractions were assayed: M/B, M*/B*, M*/B* + 5 mM caffeine, M*/B* + 200 nM wortmannin, M*/B* + ATM Ab, and M/B/B*.

(B) The levels of proteins were monitored by Western blotting in the fractions M/B, M*/B*, or M*/B* + ATM Ab as indicated above each lane. Total fractions were probed with antibodies specific against Cdk2 (top panel) and Cyclin E (second panel). Cdk2 immunoprecipitates were probed with Cyclin E antibodies (third panel), with Xic1 antibodies (fourth panel), or with phospho-tyr15-specific antibodies (bottom panel).

(C) Genomic DNA replication was monitored by incorporation of α -³²P-ATP into 6-DMAP chromatin supplemented with M and B fractions (M/B), M* and B* (M*/B*), M* and B* in the presence of recombinant Cdk2WT/CyclinE (M*/B* + Cdk2WT), or Cdk2AF/CyclinE (M*/B* + Cdk2AF) complexes at levels equivalent to 2-fold the endogenous Cdk2 kinase of M/B. The lower panel shows that identical amounts of Cdk2 kinase activity were added to M*/B* fractions.

(D) Genomic DNA replication was monitored by incorporation of α -³²P-ATP into 6-DMAP chromatin supplemented with M and B fractions (M/B), M* and B* (M*/B*), M* and B* in the presence of recombinant wild-type Cdc25A (M*/B* + Cdc25A/WT), catalytically inactive Cdc25A (M*/B* + Cdc25A/C432A), or wild-type Cdc25C (M*/B* + Cdc25C/WT).

(E) The binding of Cdc45 to the chromatin was followed by Western blotting of purified chromatin incubated in M* and B* fractions (M*/B*) or in M* and B* fractions treated with 10-fold excess of recombinant wild-type Cdk2/CyclinE complexes (M*/B* + Cdk2/CycE).

have affinity for DNA ends (Smith et al., 1999). We propose that, upon addition of DSB-containing DNA, a fraction of ATM is activated and subsequently cosediments with DNA during the fractionation procedure. ATM stably activates downstream effectors that can signal even in the absence of the checkpoint signal. This might explain why B* is dominant over B and why ATM antibodies cannot abrogate the checkpoint when added to B* after the signaling has been initiated (data not shown). Studies are in progress to analyze the DNA structures and modifications that activate the DNA damage checkpoint and to characterize the responsible protein-DNA interactions.

The Checkpoint Response Is Rapid

Signaling following DNA damage must act rapidly to prevent the replication of damaged templates at the onset of S phase. The signal must also persist to allow for repair of the damage to be coordinated with cell cycle progression. In vertebrates, a major DNA damage

response that prevents S phase entry is the p53-dependent pathway. Activation of p53 induces apoptosis or p21-mediated cell cycle delay. Cell cycle response to p53 requires transcription and de novo protein synthesis of p21, in addition to a cascade of protein modifications, including ATM/ATR and Chk2 protein kinases. The p53 pathway takes several hours to induce cell cycle arrest.

We have described a DNA damage checkpoint in an extract from a vertebrate organism that does not require the transcriptional function of p53. First, transcription in *Xenopus* embryos cannot be detected until after the midblastula transition and, second, the cell-free system we describe contains cycloheximide and cannot support protein synthesis. *Xenopus* eggs and extracts do contain p53; however, we cannot exclude a nontranscriptional function for p53.

The signaling pathway described above prevents S phase entry in 15 min. This represents the time necessary for fragmented DNA to induce inhibition of Cdk2/CyclinE. Thus, this checkpoint entails only posttranslational protein modification and is, in principle, readily

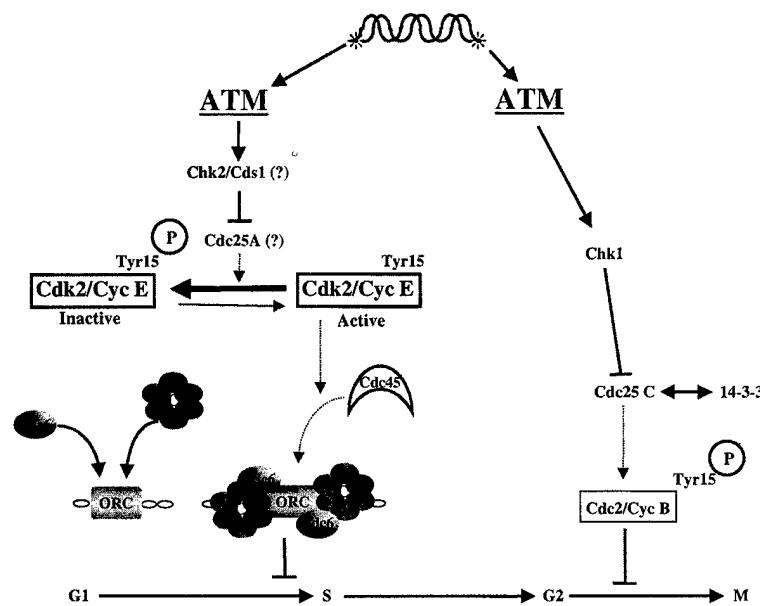


Figure 7. A Model for the ATM-Dependent Inhibition of DNA Replication Initiation following DNA Damage

Following the detection of double-strand breaks in G1, S, or G2 phases, ATM protein is activated. Prior to S phase, activation of ATM promotes a cascade of events leading ultimately to the inhibition of Cdk2/CyclinE kinase activity. We propose that ATM could activate a Chk protein (possibly Chk2/Cdcs1/Rad53), which in turn would inhibit the activity of Cdc25A. This would lead to the accumulation of inactive Cdk2/CyclinE complexes in which Cdk2 is phosphorylated on tyrosine 15. The inhibition of Cdk2 activity prevents the loading of Cdc45 on the chromatin, an essential late step in the assembly of the pre-RC.

reversible. DNA damage checkpoints at other cell cycle stages with similar characteristics have been described. These inactivate cyclin-dependent kinases by phosphorylation. A checkpoint that modifies the tyrosine 15 of Cdc2 operates at the G2/M border (Furnari et al., 1997; Sanchez et al., 1997). A second checkpoint induced by UV irradiation functions in G1 and results in the phosphorylation of tyrosine 17 of Cdk4 (Terada et al., 1995).

A Conserved DNA Damage Checkpoint

DNA damage checkpoints that prevent DNA replication in a p53-independent manner have been described in a variety of organisms. Recall that budding yeast does not contain a p53 homolog but is subject to S phase arrest by DNA damage. Rad9 may play a role in this response (Siede et al., 1993). DNA damage in mammalian cells inhibits S phase entry or ongoing replication in a p53-independent manner. Inhibition of S phase entry in CHO p53^{-/-} cells takes place within 2 hr following DNA damage and coincides with 30% inhibition of Cdk2/CyclinE activity (Lee et al., 1997; Xie et al., 1998). Similar to our findings, it was recently reported that UV irradiation induces downregulation of Cdc25, leading to the accumulation of inactive Cdk2/CyclinE phosphorylated on tyrosine 15 (Mailand et al., 2000). Finally, the RDS phenotype we reconstituted *in vitro* is not only reminiscent of the ATM cellular phenotype but also of that of ATLD (ataxia-telangiectasia-like disease) phenotype. ATLD cells harbor mutations in the *hMRE11* gene but display normal p53 activation (Stewart et al., 1999). Taken together, these observations suggest that a checkpoint similar to the one we describe in *Xenopus* cell-free extracts may operate in mammalian cells.

Inhibition of X-ATM was sufficient to abrogate the checkpoint and to restore DNA replication to levels similar to controls. This suggests that ATM is the major protein operating in this pathway and allows us to rule out possible redundancy between ATM and ATR in the response to DSB-containing DNA. We cannot rule out, however, that ATR might function in a linear path with

ATM or might operate in other signaling pathways activated by different types of damages.

Although we have shown that ATM lies upstream of Cdk2/CyclinE in the same linear pathway, we do not believe that ATM directly regulates Cdk2 activity. By analogy with the DNA damage checkpoint that inactivates Cdc2/CyclinB at the onset of mitosis, we propose that ATM might act through Chk2 kinase and Cdc25A (Figure 7), since DSBs have been shown to promote phosphorylation of Chk2 (Guo and Dunphy, 2000). Clarification of these signaling steps awaits further investigation.

Inhibition of DNA Replication

We have analyzed the effect of fragmented DNA on pre-RC assembly. The assembly of ORC, Cdc6, the MCM proteins, and Cdc7 on chromatin was resistant to checkpoint control. Activation of pre-RC requires the activity of Cdk2, Cdc7, and PKA protein kinases. Cdc7 is a cell cycle-regulated protein kinase that is essential for S phase entry in *Xenopus* and other organisms (Sclafani and Jackson, 1994; Jiang et al., 1999; Roberts et al., 1999). Cdc7/Dbf4 protein kinase is the target for a DNA replication checkpoint following exposure to hydroxyurea in budding yeast (Weinreich and Stillman, 1999). We did not observe alterations in the binding of Cdc7 to chromatin following activation of the DNA damage checkpoint. In addition, we have shown that the replication defect associated with DNA damage checkpoint activation could be fully rescued by addition of Cdk2/CyclinE protein kinase.

We showed that downregulation of Cdk2/CyclinE by phosphorylation on tyrosine 15 was necessary to prevent DNA replication following DNA damage. First, changes in the levels of endogenous Cdk2 protein kinase activity followed exactly the changes in levels of DNA replication in all experimental conditions tested. Second, 2-fold overexpression of recombinant Cdk2AF/CyclinE, but not Cdk2WT/CyclinE, could rescue the ability of an extract with an activated checkpoint to undergo DNA replication. Third, addition of recombinant Cdc25A

could also rescue DNA replication in similar conditions. Ten-fold overexpression of exogenous Cdk2WT/CyclinE in B^{*}/M^{*} rescued DNA replication after activation of the checkpoint by restoring Cdk2 kinase to levels comparable to B/M. The overexpression of purified Cdk2/CyclinE complex allowed the inhibitory signal contained in fraction B^{*} to be overcome, restoring the activity to the level of B/M. Interestingly, addition of Cdk2/CyclinE (WT or AF) was only able to rescue DNA replication when added following the assembly of the pre-RC on the chromatin. Addition of Cdk2/CyclinE prior to pre-RC assembly did not rescue replication probably due to the inhibitory effect of Cdk2 on pre-RC assembly (Hua et al., 1997).

We showed that inhibition of Cdk2/CyclinE was accompanied by the inability of Cdc45 to bind to the chromatin (Figure 7). This is consistent with the observation that Cdc45 fails to bind to chromatin in extracts depleted of Cdc2 and Cdk2 by p13^{sucl} beads (Mimura and Takisawa, 1998). Loading of Cdc45 on the chromatin is the last initiation event prior to the loading of the DNA polymerases. Thus, inhibiting initiation at this step provides an economical way to block DNA replication without disassembling the pre-RC.

Experimental Procedures

Cell-Free Extracts

Extract Preparation

CSF-arrested extracts were freshly prepared according to Murray (1991). For replication assays, extracts were supplemented with 100 µg/µl of cycloheximide and released in interphase with 0.4 mM CaCl₂. 6-DMAP (Sigma) extract was prepared in a similar way, except that 3 mM 6-DMAP was added prior to CaCl₂ addition.

Checkpoint Extracts

Uncut plasmid DNA, digested plasmid, or λ phage DNA were incubated in interphase extracts for 15 min to activate the checkpoint. For the rescue experiment, extracts were pretreated with 5 mM caffeine, 200 nM wortmannin, or affinity-purified anti-X-ATM antibodies (Robertson et al., 1999) following calcium addition and then incubated with damaged DNA.

Extract Fractionation

Fractions were prepared as originally described by Chong et al. (1997) to study DNA replication initiation. Interphase extracts treated as described above were subjected to fractionation to yield fractions M and B. Briefly, extracts were diluted 4-fold with cold LFB buffer (50 mM KCl, 40 mM HEPES KOH [pH 8.0], 20 mM K₂HPO₄/KH₂PO₄ [pH 8.0], 2 mM DTT, 2 mM MgCl₂, 1 mM EGTA, 10% sucrose, 1 µg/µl of aprotinin, pepstatin, and leupeptin). They were subjected to an ultracentrifugation step at 80,000 × g for 40 min at 4°C, using a swing out rotor. Supernatants were carefully transferred to avoid pellet contamination and supplemented with 0.075 vol of a 50% PEG solution to give a final concentration of 3.5%. Samples were incubated on ice for 30 min and spun for 10 min at 10,000 rpm at 4°C. Pellets were resuspended in LFB supplemented with 2.5 mM Mg-ATP at 5× concentration with respect to undiluted extract. This pellet corresponded to fraction B. The corresponding supernatant was adjusted to 9% PEG, and proteins were precipitated as before to yield fraction M.

DNA Templates

Chromatin Templates for Replication

Demembranated *Xenopus* sperm nuclei were prepared as described (Murray, 1991) and frozen in aliquots in liquid nitrogen. 6-DMAP chromatin was assembled as follows: 40,000 nuclei/µl. were incubated for 20 min at 23°C in 6-DMAP extract. The extract was then diluted 10-fold in chromatin isolation buffer (50 mM KCl, 5 mM MgCl₂, 2 mM DTT, 50 mM HEPES, 0.5 mM spermine 3HCl, 0.15 mM spermidine 4HCl, 1 µg/µl aprotinin, pepstatin, leupeptin, and 0.01% Triton X-100), underlayered with the same buffer containing 30% sucrose. Chromatin was then pelleted at 6000 × g for 15 min at 4°C

and was resuspended in chromatin isolation buffer supplemented with 2.5 mM Mg-ATP.

DSB-Containing Templates

To prepare molecules containing double-strand breaks, we used circular pBR 322 plasmid that was digested with restriction endonucleases to yield different types of ends (3'-, 5'-, and blunt). Since no difference was noticed with different ends, HaeIII digestion was used to provide a high number of ends. To obtain increasing number of ends, λ phage DNA was digested with XbaI, HindIII, NcoI, BstE, or HaeIII.

Replication Assays

Replication assays were performed by mixing 0.5 µl of 6-DMAP chromatin (10,000 nuclei/µl) with 1 µl of each M and B or M^{*} and B^{*} fraction obtained from extract treated with different types of DNA molecules. The reaction was incubated for 15 min at 23°C. Ten microliters of 6-DMAP extract was then added, and DNA synthesis was monitored by the incorporation of α -P³²-dATP for 90 min at 23°C following agarose gel electrophoresis (Costanzo et al., 1999). For Cdk2/CyclinE rescue experiments, fractions B^{*} and M^{*} were incubated for 30 min with 6-DMAP chromatin, and recombinant Cdk2/CyclinE (Hendrickson et al., 1996) was added to the reaction at 20 min.

Antibodies and Protein Assays

Chromatin Binding Assays

For chromatin binding assays, replication reactions were assembled as above, except they were scaled up 10-fold. Ten microliters of M and B fractions were incubated for 15 min with 5 µl of 6-DMAP chromatin (10,000 nuclei/µl). Following incubation, each reaction was diluted in 200 µl of chromatin isolation buffer supplemented with 0.1% Triton X-100 and underlayered with the same buffer containing 30% sucrose. The chromatin was pelleted at 6000 × g for 15 min at 4°C. The pellet was resuspended in Laemmli loading buffer. The samples were run on 10% SDS-PAGE and analyzed by Western blotting with ORC2, CDC6, MCMs, CDC7, and CDC45 polyclonal rabbit antibodies. Antibodies against ORC2, MCM3, MCM5, MCM7, and Cdc6 were a generous gift from Dr. Laskey, Dr. Madine, and Dr. Romanowski and were used as previously described (Madine et al., 1995; Hendrickson et al., 1996; Romanowski et al., 1996; Sible et al., 1998). Anti-Cdc7 was used as described previously (Roberts et al., 1999). Antibodies against XMCM4 were described previously (Hendrickson et al., 1996). Antibodies against MCM2 and MCM6 were generated against the full-length recombinant XMCM2 and XMCM6 expressed in baculovirus-infected cells. Antibodies against XCdc45 were a generous gift from Dr. Takisawa.

Cdk2 Kinase Assays

Immunoprecipitation of Cdk2 kinase activity was performed as follows: 10 µl of M and B fractions or M^{*} and B^{*} was incubated with 5 µl of 6-DMAP chromatin for 15 min at 23°C. The reactions were diluted in PBS supplemented with protease inhibitors and 0.2% Triton X-100 buffer to a final volume of 250 µl. The samples were precleared with protein A-Sepharose, incubated with affinity-purified rabbit Cdk2 antibody on ice for 2 hr, mixed with 25 µl of 50% protein A-Sepharose for 1 hr, washed with low and high salt buffers, and washed with EB buffer. Immunoprecipitates were incubated with EB buffer supplemented with 0.5 mg/ml histone H1 and 50 µM ATP and 1 µl of γ -P³²-ATP, 10 mCi/ml (>3000mC/µl). Samples were incubated at 25°C for 20 min and reaction stopped by the addition of 25 µl of 2× Laemmli buffer. The reactions then were boiled and electrophoresed on 12.5% SDS-PAGE.

Western Blot Analysis

Samples from M/B and M^{*}/B^{*} replication reactions were diluted in loading buffer, boiled for 3 min, electrophoresed, transferred to nitrocellulose, and probed with polyclonal antibodies specific for *Xenopus* CycE (Rempel et al., 1995), Xic1 (Su et al., 1995), and Cdk2 (Gabrielli et al., 1992). Immunoprecipitates with anti-XCdk2 were blotted with CycE antibodies and human Cdk2 antiphospho-Tyr-15 (New England Biolabs).

Cdk2 antibodies were raised against the C-terminal 16-amino acid of *Xenopus* Cdk2 (Gabrielli et al., 1992). Antibodies against Cyclin E and Xic1 were a generous gift from Dr. J. Maller.

Production of Recombinant CDK2 AF Mutant

Cdk2AF mutant was generated by PCR using a 59-mer 5' mutagenic primer complementary to the 5' region of hCdk2 ORF containing

base substitutions replacing threonine 14 by alanine, and tyrosine 15 by phenylalanine and an EcoRI restriction site. The 3' primer contained an Xhol site: 5'-GCGCGAATTCTGGAGAACTTCAA AAGGTGAAAAGATCGGAGAGGGCGCGTTCGGAG; 3'-GCGCT CGAGTCAGAGTCGAAGATGGGGTACTGGC.

The PCR product was sequenced and subcloned into *pFastBac1* (GIBCO) using the EcoRI and Xhol sites. Baculovirus expression system BacToBac (GIBCO) was then used to generate viral genomic DNA encoding for Cdk2AF. Sf9 cells were transfected with viral genomic DNA carrying Cdk2AF, and viruses were harvested from the medium and used for subsequent infections.

Expression and Purification of Proteins from Insect Cells

Active Cdk2AF/CyclinE and Cdk2/CyclinE complexes were purified from Sf9 cells according to Harper et al. (1995). Briefly, 150 ml of Sf9 cells (1×10^6 cells/ml) were infected with Cdk2AF virus or Cdk2WT virus along with GST-CyclinE virus. After 48 hr, cells were harvested and lysed in buffer containing 20mM Tris-HCl (pH 8.0), 2 mM EDTA, 100 mM NaCl, 5 mM NaF, 30 mM p-nitrophenylphosphate, 1 mM PMSF, 0.5% Nonidet P-40 (Harper et al., 1995). The lysate was diluted 4-fold with 20 mM HEPES (pH 7.5), 15 mM MgCl₂, 5 mM EGTA, 1 mM DTT, 1 mM ATP, and protease/phosphatase inhibitors were incubated with 1 ml of glutathione-Sepharose (Pharmacia) for 60 min. The resin was extensively washed, and GST-CyclinE/Cdk2WT or GST-CyclinE/Cdk2AF was eluted with 2 ml of 50mM Tris-HCl (pH 8.0) containing 20 mM glutathione, 120 mM NaCl plus protease and phosphatase inhibitors.

Xenopus Cdc25A, Cdc25A catalytic inactive mutant (C432A), and Cdc25C were purified according to Izumi and Maller (1993). Cdc25-expressing baculoviruses were a gift from Dr. J. Maller.

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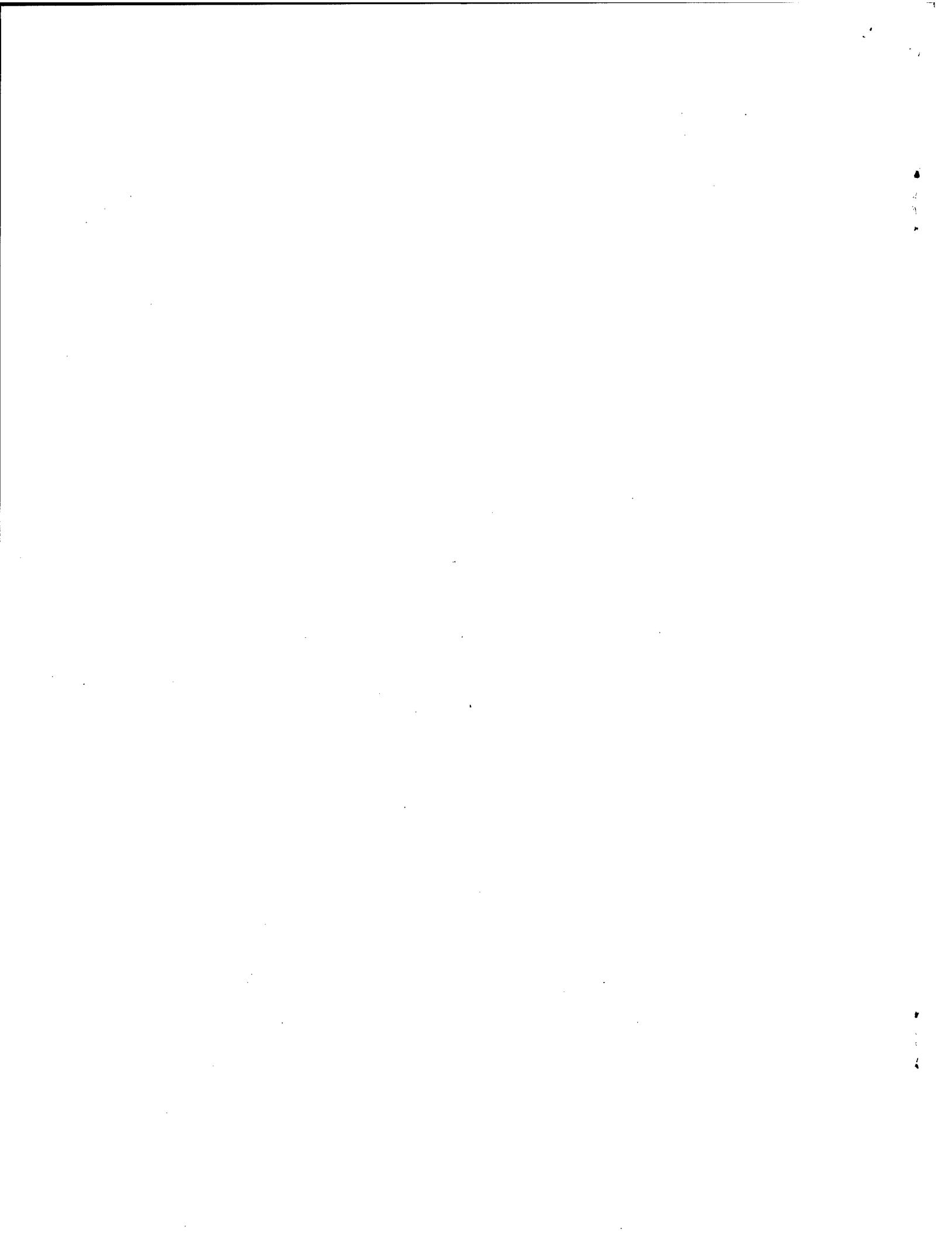
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Responses to DNA damage in *Xenopus*: cell death or cell cycle arrest

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Abstract. *Xenopus* embryos divide rapidly following fertilization. During this rapid period of cleavage, cell divisions are not sensitive to DNA replication or spindle assembly inhibition. Here, we have investigated the consequences of eliciting DNA damage in these embryos. We show that the rapid cell divisions are not affected by DNA damage. However, as the embryos reach the onset of gastrulation, they undergo rapid and synchronous apoptosis. We have investigated the regulation of this delayed apoptotic response to DNA damage. Next, we have reconstituted a DNA damage cell cycle checkpoint *in vitro*, demonstrating that all the checkpoint signalling components are present in the embryos but are not activated under the experimental conditions used to generate DNA damage in the embryo.

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DNA damage events that can produce transmissible modifications in the genome are under surveillance in order to prevent their propagation. These surveillance mechanisms work through a network of signal transduction pathways that can either prevent cell cycle progression or alternatively result in the induction of cell death (Elledge 1996, Hensey & Gautier 1995).

During the cell cycle genomic DNA is replicated during S phase and then distributed to the daughter cells as chromosomes during mitosis. Replication should faithfully duplicate the genetic material, take place only once per cell cycle and always occur between intervening mitoses to maintain the characteristic ploidy of the genome. Replication should use undamaged templates, so that the information transmitted to the daughter cells does not get modified throughout cell generations. In a similar manner, mitosis should also segregate identical pools of undamaged chromosomes. Therefore, the decisions to start DNA replication or to start mitosis, the G/S and G2/M transitions, are under tight regulation and surveillance.

The surveillance mechanisms that prevent cell cycle progression following DNA damage operate throughout the cell cycle, as it has been shown that

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damage can be sensed in G1, S or G2 phases. It has been proposed that cell cycle delay allows for the coordinated repair of damaged DNA. Alternatively, cells that have undergone DNA damage can be eliminated by apoptotic mechanisms. It appears that cell cycle arrest could also be a first step towards the expression of a cell death program as some of the critical regulators of cell cycle arrest such as p53 are also essential for the occurrence of cell death.

We have started to investigate these two types of responses to DNA damage in *Xenopus* eggs and embryos, and cell-free extracts derived from them. Early work had suggested that *Xenopus* embryos lacked some of the surveillance mechanisms or cell cycle checkpoints that are normally operating in somatic cells. For example, experimental interference with DNA replication using aphidicolin (an inhibitor of DNA polymerase), or with mitotic spindle formation using microtubule poisons, did not prevent cell cycle progression in *Xenopus* embryos as seen by the continuing oscillations of the mitotic kinase Cdc2/cyclin B in treated embryos (Kimelman et al 1987, Newport & Kirschner 1982). Since then, it has been accepted that *Xenopus* embryos lacked all types of cell cycle checkpoints. In this work, we have more carefully looked at the consequences of inducing DNA damage on the early cell cycles.

Results and discussion

*Induction of DNA damage to the cleaving *Xenopus* egg does not block cytokinesis*

We used two different experimental means for inducing DNA damage in *Xenopus* eggs during cleavage. Fertilized eggs were injected at the one or two cell embryo stage with restriction enzymes, or embryos were subjected to γ -irradiation at various stages following fertilization. In either case, we did not observe a delay in cell cycle progression when compared to control uninjected embryos (Hensey & Gautier 1997).

Cell cycle progression in treated eggs was monitored by live video microscopy following both the cleavage of the egg into blastomeres and the cortical contraction waves preceding cytokinesis. One example of such experiments is presented in Fig. 1A as still shots of a time-lapse video recording of a control untreated egg alongside an egg irradiated with 40 Gy one hour following fertilization. No difference in the timing of cell divisions was observed up to the 12th cleavage (Fig. 1A), the time of the midblastula transition (MBT). At the MBT, cell divisions normally become asynchronous in *Xenopus* embryos (Newport & Kirschner 1982). We observed this developmentally regulated cell cycle transition in untreated embryos. Surprisingly, irradiated embryos underwent another two synchronous cleavages as seen by both the progression of cytokinesis and by the contraction waves in time-lapse video microscopy. In

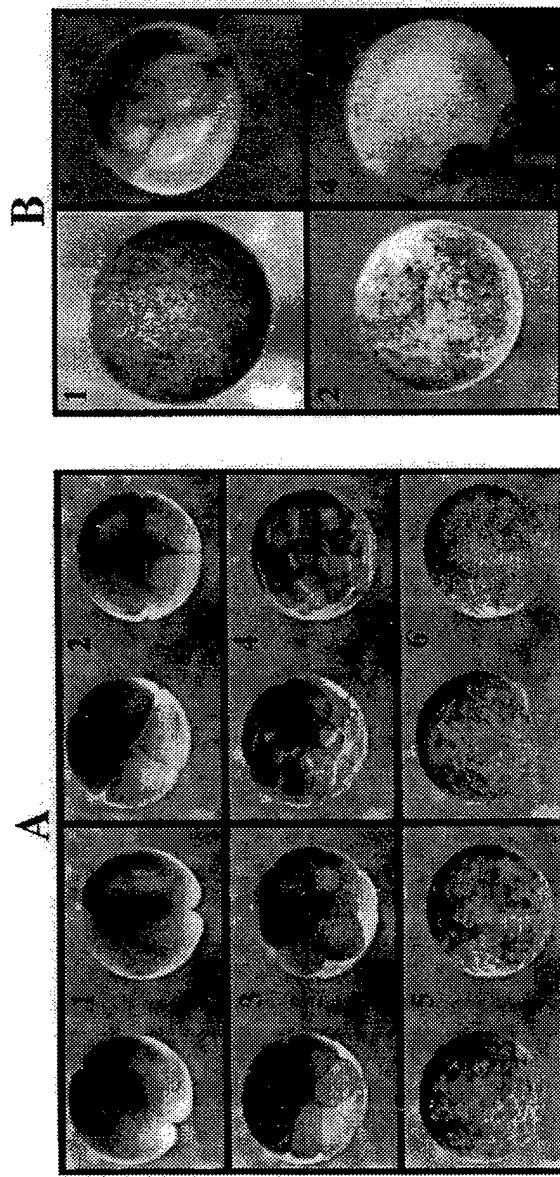


FIG. 1. Induction of synchronous cell death in embryos. Early cell divisions were monitored by time-lapse video recordings. (A) Panels 1–6 compare a control (right), and irradiated (left) embryo. Panel 1, St. 2; panel 2, St. 3, panel 3, St. 5; panel 4, St. 7; panel 5, St. 8; panel 6, St. 9. (B) Panel 1, control embryo, St. 10.5; panel 2, dying irradiated embryo, St. 10.5; panel 3, dying cycloheximide-treated embryo. This embryo was treated with cycloheximide at the 2-cell stage, at a concentration of 0.1 mg/ml which inhibited protein synthesis by 97%, cell division arrested at the 4-cell stage, and death occurred at a time equivalent to St. 10.5; panel 4, dying α -amanitin-treated embryo, St. 10.5. Death is defined as the rapid disintegration of the embryo characterized by a white mottled appearance.

addition, we followed cell cycle progression biochemically in irradiated and control eggs by monitoring the oscillation of the mitotic kinase Cdc2/cyclin B. We found no difference in the timing or the extend of the oscillation of Cdc2 protein kinase during the two cell cycles following irradiation as compared to unirradiated controls. Upon examination of the total genomic DNA content of irradiated embryos, we found that embryos that were irradiated at the one cell stage contained 80% of the DNA content of untreated embryos by the time they reached the 12th division. This established that although DNA replication was slightly impaired, the bulk of it took place in irradiated embryos. We propose that this 20% reduction in genomic DNA might be due to the early loss of broken chromosomes following the generation of double-strand breaks (DSBs) in the DNA that were not repaired before the first mitosis. A 20% decrease in genomic DNA at the time of the MBT would result in a 20% decrease in the nuclear:cytoplasmic ratio which probably accounts for the two extra synchronous divisions, i.e. for the delayed MBT observed in these embryos. Indeed, it has been proposed that the timing of the MBT is directly regulated by the nuclear:cytoplasmic ratio. Doses of γ -irradiation ranging from 20 to 200 Gy resulted in a similar phenotype. Similarly, restriction enzyme-injected embryos divided at the same speed as control uninjected embryos.

These experiments clearly demonstrated that despite the occurrence of extensive DNA damage, particularly DSBs, the embryonic cells kept progressing throughout the cell cycle unaffected, demonstrating that the checkpoint pathway that normally prevent cell progression following DNA damage was not operational in these cells.

DNA damage to the early embryos promotes a synchronous but delayed apoptotic response

When embryos treated as described above were left to develop, they reached the onset of gastrulation with no visible changes in morphology compared to untreated embryos. However, as they reached stage 10.5, the embryos underwent rapid and synchronous apoptosis. This rapid cell death had striking morphological characteristics: individual cells from the embryo lost their cohesion and adhesiveness and rapidly lysed so that few or no intact cells were found in each embryo 20 minutes following the onset of apoptosis (Fig. 1B).

This delayed apoptotic response is developmentally regulated and exhibits some striking features. Induced apoptosis could never be elicited prior to the MBT, regardless of the nature of the apoptotic inducer and of the dose, provided it was above a threshold. However, the components of the apoptotic cascade are present as early as the unfertilized eggs since extracts from these eggs can undergo genuine apoptosis *in vitro* (Evans & Kornbluth 1998). The apoptotic stimulus could only elicit the response when applied prior to the MBT (Hensey & Gautier 1997, Sibley

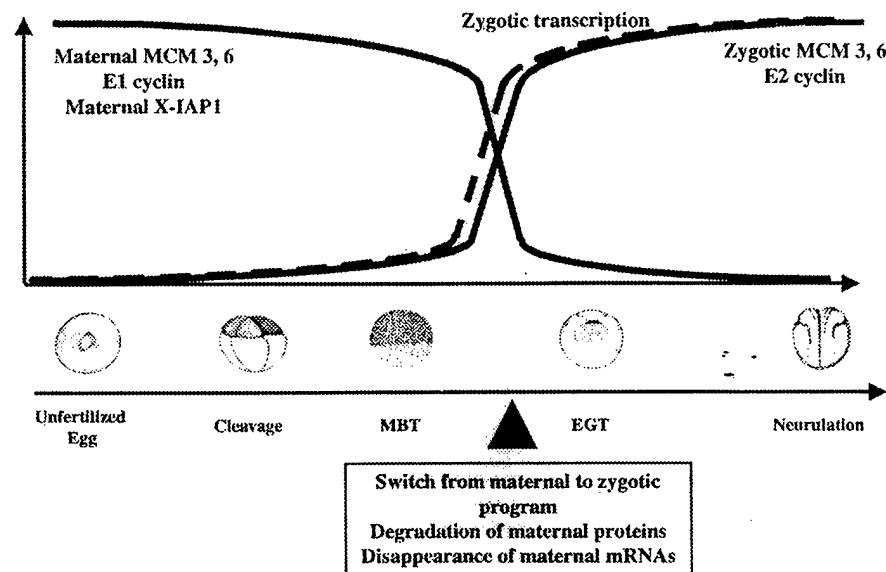


FIG. 2. Schematic representation of the switch from a maternal to a zygotic programme taking place in *Xenopus* between the MBT and the onset of gastrulation. This switch has been shown to operate for cell cycle regulators such as cyclin E and MCM proteins. We propose that maternal regulator(s) of apoptosis such as X-IAP 1 are also expressed in this kind of pattern and could explain the apoptotic program we have uncovered.

et al 1997). Finally, the apoptotic response did not require *de novo* transcription or protein synthesis. A similar programme has been identified by others in *Xenopus* and zebrafish (Hensey & Gautier 1997, Ikegami et al 1999, Sible et al 1997, Stack & Newport 1997).

These observations lead us to propose the hypothesis proposed on Fig. 2, in which the cleaving eggs contains maternal stockpile of stable protein(s) that inhibit apoptosis. Following the MBT and prior to the onset of gastrulation, these proteins become highly unstable leading to apoptotic death of the embryo unless a zygotic form of the inhibitor is synthesized. When this synthesis is prevented experimentally by either inducing DNA damage, blocking transcription or blocking protein synthesis, apoptosis takes place.

Search for maternal factors that can modulate the apoptotic response

In order to identify maternally inherited factors that regulate this apoptotic response, we used a screening strategy depicted in Fig. 3. We expressed pools of mRNAs, transcribed from a stage 4 maternal library (Lagna et al 1999). Each pool

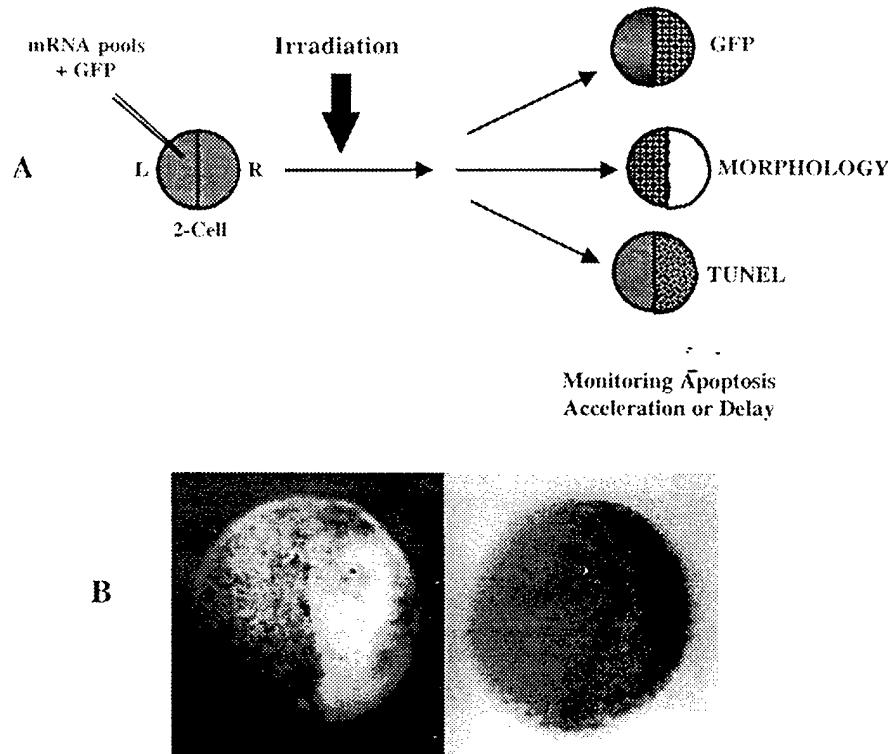


FIG. 3. (A) Screening scheme for maternal genes that can modulate apoptosis when expressed in cleaving embryos. One cell of a two-cell stage embryo is injected with GFP and the expression library (see text). The injected embryos are irradiated and score for delay or acceleration of apoptosis in the injected half. (B) The example of *BCL2* injection in this screen is shown. Left panel shows embryonic morphology, right panel shows TUNEL stained embryos. Both panels show a St. 10.5 embryo, cells in the left half of the embryo, i.e. those arising from the blastomere injected with *BCL2* RNA, look normal while the right half of the embryo is dying.

contained 100 clones. Each pool was tested for its ability to delay or accelerate apoptosis when injected in once cell of a two cell stage embryo. The recipient embryo is induced to undergo apoptosis at stage 10–10.5 by γ -irradiation at the two cell stage. The embryos were screened for morphology as well as processed for whole mount TUNEL staining. The injected side was marked by co-injecting mRNAs encoding for green fluorescent protein (GFP) (Fig. 3A). In such a screen, an activity will be uncovered when the injected half undergoes apoptosis either late or early, as compared to the uninjected side. A visual example of the assay is given in Fig. 3B using *BCL2* mRNA to delay apoptosis in the injected half. When a pool of mRNAs displayed activity, the cDNA responsible for the effect observed was

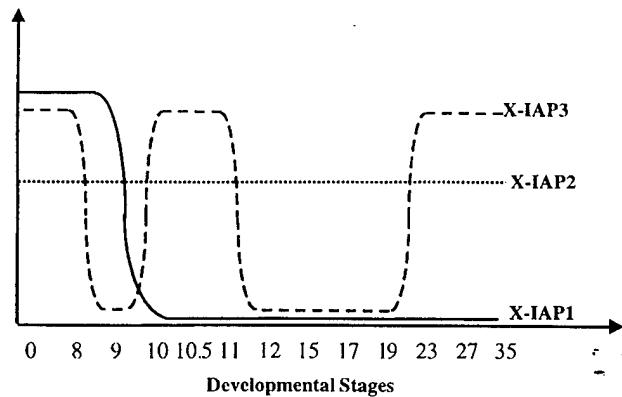


FIG. 4. Temporal expression pattern of three *Xenopus* IAP family members. We have cloned three IAPs designated X-IAP1, 2 and 3. This graph shows their developmental profile as assessed by RT-PCR.

isolated by sib-selection (Smith & Harland 1992). To date, we have not isolated clone that accelerated apoptosis, confirming the hypothesis that maternal factors are preventing cell death in the early embryos. However, we have isolated two clones that delay apoptosis to a similar extent as human *BCL2* mRNA does. Interestingly, one clone delays apoptosis induced by either irradiation or transcription inhibition while the other delays apoptosis induced by irradiation only. The expression and characterization of the role of these molecules in regulating apoptosis is under current investigation.

In a second approach, we cloned *Xenopus* homologues of the inhibitor of apoptosis gene family (IAPs), a conserved family of potent inhibitors that can block cell death at different steps of the apoptotic cascade (Deveraux & Reed 1999). In *Drosophila*, a maternal IAP has been recently implicated in regulating cell death early during development (Wang et al 1999). So far, we have isolated three different *Xenopus* genes encoding for IAP family members. All three are maternally inherited, and two of them are also transcribed later during development as shown on Fig. 4. Interestingly, the clone designated X-IAP1 is exclusively maternal and is rapidly degraded between the MBT and the onset of gastrulation, fulfilling the criteria for being a bona fide maternal regulator of the apoptotic programme.

*Reconstitution of a DNA damage checkpoint in extracts derived from *Xenopus* eggs*

DNA damage in the context of the embryo does not inhibit cell division as seen by the synchronous and timely occurrence of cytokinesis. In a similar fashion as a

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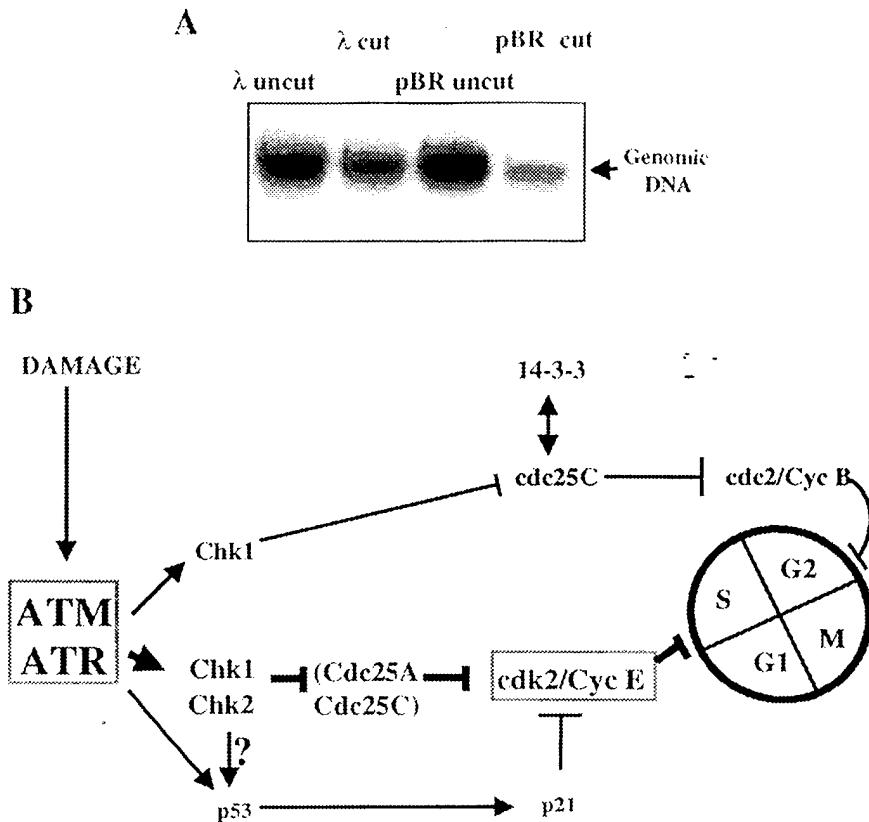


FIG. 5. DNA damage cell cycle checkpoint in *Xenopus* extracts. (A) Genomic DNA replication was measured using intact chromosomal templates in different extracts. Extracts were incubated with either plasmid or X, DNA, intact or digested, as indicated. In presence of digested DNA that contained double strand ends, genomic replication was inhibited. (B) A model for the ATM-dependent inhibition of DNA replication initiation following DNA damage. Following the detection of double strand breaks in G1, S or G2 phases, ATM protein is activated. Prior to S-phase, activation of ATM promotes a cascade of events leading ultimately to the inhibition of Cdk2/cyclin E kinase activity. By analogy with the ATM-dependent signalling taking place in G2, we propose that ATM could activate a Chk protein (possibly Chk2/Cdc1/Rad53), which in turn would inhibit the activity of Cdc25. This would lead to the accumulation of inactive Cdk2/cyclin E complexes in which Cdk2 is phosphorylated on tyrosine 15.

cell cycle checkpoint was uncovered in extracts following inhibition of DNA replication (Dasso & Newport 1990), we investigated whether it was possible to elicit a cell cycle response following DNA damage *in vitro*. We decided to use initiation of DNA replication as a readout for monitoring DNA damage checkpoint activation. It is not really understood how DNA damage is

recognized when a cell cycle checkpoint is activated and what type of DNA structures can elicit such responses. It has been proposed that DNA breaks themselves could be recognized (single or double strand breaks), or single strand region of DNA. In either case, molecules with affinity for breaks or single strand DNA would be involved and these molecules could also be essential for DNA replication. To circumvent this problem we established an *in vitro* system in which we monitored the activation of a DNA damage checkpoint *in vitro*. DNA molecules containing double-strand breaks (DSBs) ~~or not~~ were introduced into a *Xenopus* interphase extract and signalling was allowed to take place for 15 min. The extract was then fractionated (Chong et al 1997) and the fractions were reassembled into a cell-free system in which DNA replication initiation of undamaged chromosomal DNA was monitored. In this system, the DNA used to elicit the signalling is eliminated during the fractionation procedure.

Using this system we showed that DSBs-containing DNA can elicit a checkpoint as seen by the inhibition of DNA replication of intact genomic templates (Fig. 5A). Undamaged DNA (circular plasmid or undigested lambda DNA) did not elicit the checkpoint. Using specific drugs that inhibit ATM (the product of the gene mutated in ataxia-telangiectasia) and the ATM family of protein kinases (Jeggo et al 1998) as well as antibodies specific for ATM (Robertson et al 1999) we showed that the checkpoint is ATM dependent (Fig. 5B). Finally, we showed that the checkpoint leads to the inactivation of Cdk2/cyclin E protein kinase which is essential for DNA replication (Fig. 5B).

In summary, we showed that DNA damage can elicit a variety of responses in *Xenopus* early embryos. While γ -irradiation induces apoptosis in the embryos, DSB-containing DNA prevents initiation of DNA replication in cell-free extracts derived from eggs.

Acknowledgements

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Author reference
Wang et al 1999
should
Drosophila be
italics?

YES

DISCUSSION

Maller: We find a Bcl2 band in the pre-MBT embryo that doesn't change in abundance after the MBT. If we put in Bax, which promotes apoptosis, we can pull out that Bcl2. In addition to the maternal IAPs that you got, there is maternal Bcl2 preventing apoptosis before the MBT.

Gautier: That is why we have this biased approach with IAPs, because there are data in *Drosophila* showing that there is a maternally inherited inhibitor of apoptosis. We are also screening for a maternally inherited molecule that can affect the onset of apoptosis. These could be regulating apoptosis directly. Also, as John Newport mentioned, I tend to believe that it might be some sort of more general mechanism linking all these maternal zygotic switches through degradation of mRNA.

Maller: I was surprised that you could remove the damaged DNA and retain the signal for all these manipulations. You would have thought that removing the

DNA would be analogous to having repaired all the damage, and everything should come back to equilibrium.

Gautier: It looks like the modification of the signalling is dominant and stable.

Maller: This almost implies that the repaired DNA would have to send a signal back saying that now everything is OK.

Gautier: It is a very stable signal.

Nurse: In relation to that, after having sent the signal with broken DNA, if you now put the good DNA back, would you reverse it?

Gautier: There is genomic DNA in the assay and such reversal doesn't take place. It is clear that a circular plasmid doesn't do that, even at a tenfold higher level. So checkpoint signalling is dependent on DNA ends.

Nurse: Does this mean that it is the repair process itself is what is removing the signal?

Maller: Yes, it suggests this. More than that, it says that the repaired product must send a signal back. You have removed the damaged signal by centrifugation.

Gautier: That is clear. We cannot see any DNA in any of the B or M fractions. There is a stable modification that is induced by DNA breaks in the B fraction.

Nasmyth: Is that in addition to undamaged B? Is it dominant?

Gautier: It is dominant. If we mix B, M and B* there is a checkpoint. This is another way of showing that this is real signalling.

Harper: Can you bypass the checkpoint by using a Cdk2AF mutant? The simplest hypothesis is that you are phosphorylating Cdc25A, it is not active and you can accumulate phosphorylated Cdk2, which is just blocking activity.

Gautier: I don't know.

Harper: Steve Jackson's lab has reported that ATM binds to DNA ends as visualized by SEM. This would be consistent with you bringing down a certain amount of ATM.

Edgar: I am still puzzled with the relationship between what you found *in vivo* and what you did in the extracts. How can you reconcile having a checkpoint in the extracts but not in the animal?

Gautier: There are two possible explanations. The trivial one is that it is a problem of dose, in that in the embryo the right number of breaks is not reached, because it is a fairly large amount of cytoplasm and the signalling is too weak. The other option is that there is no replication checkpoint. Blocking replication in the embryo will therefore not affect the G2/M transition. The embryo will still divide even though it doesn't replicate.

Edgar: That should be obvious if you just stain those irradiated MBT embryos with DAPI.

Nasmyth: They go on dividing not just for the next division but for multiple divisions. This is inconceivable.

Gautier: There is replication. We have looked at the amount of DNA, and there is replication. I suspect it is more likely to be a problem of dose. We need to reach a threshold of DNA ends in order for activation to occur.

Nasmyth: What dose of radiation do you use?

Gautier: In the embryo we get a uniform cell death at about 20 Gy.

Maller: We have gone as high as 50 and don't see any difference.

Gautier: We have been to 200 Gy and still the cells die at the same time.

Raff: Is it known what the zygotic inhibitor might be that takes over from the maternal one? Is there a Bcl2 family member that could explain the zygotic switch?

Gautier: There is a Bcl-like zygotic member that Tata originally cloned.

Raff: Jim Maller, I know you found some Bcl2 family members after MBT. Could they be the answer?

Maller: Tata reported that Bcl2 mRNA only came up after the MBT. Our default model is that the cell will apoptose unless it produces a zygotic inhibitor. We thought this was Bcl2, but we have now made an antibody against it and there is lots of Bcl2 in the pre-MBT embryo. It is functional in the sense that you can get effects on apoptosis by chelating it with things like Bax. This means that there will be multiple inhibitors in the embryo. We haven't been able to clone a maternal form of Bcl2, but this does not mean that it isn't there.

Raff: Which Bcl2 family members turn on transcriptionally at MBT?

Maller: There is no change in the blottable abundance of Bcl2.

Raff: Is most of that maternal?

Maller: The maternal and zygotic gene products are immunologically indistinguishable. We see the message increase with zygotic transcription but no change in the protein. This also occurs for Cdc2, for example. You degrade maternal Cdc2 mRNA at the MBT without changing Cdc2 protein levels on blots.

Raff: So we don't know what the putative zygotic inhibitor is?

Gautier: No. There could be more than one. The only thing we need to make as a hypothesis is that there is a switch from the maternal to the zygotic whatever it is, and then at some point, if you just block the zygotic transcription, the default pathway is cell death.

Edgar: Has anyone done an experiment that results in apoptosis earlier than the MBT?

Gautier: That is what we would like to do. I mentioned that when you increase the dose of protein synthesis inhibitor or irradiation up to 200 Gy, the onset is not moved. One of the experiments to do is attempt to deplete the maternal store of IAPs, to see whether the cells enter apoptosis more rapidly.

Maller: You can do the same kind of experiment with Bax and functionally remove all maternal Bcl2 in the embryo. Apoptosis is still not initiated until after the MBT.

Gautier: This doesn't mean necessarily that there is no inhibitor present.

Maller: Yes, there could be another inhibitor, but Bcl2 is pretty powerful as far as controlling apoptosis goes. You might expect the removal of Bcl2 to have changed the timing of apoptosis if that was possible.

Nurse: If you inject DNA at the early stage, do they then undergo apoptosis?

Gautier: We have never done the experiment, but if you modulate the content of genomic DNA, you change the onset of the MBT. We don't know what happens as far as apoptosis is concerned. If the timing mechanism that controls apoptosis is similar to the one that controls the destruction of cyclin A, we would predict that this wouldn't change because the onset of cyclin A degradation doesn't seem to be dependent on when the MBT is taking place.

Lehner: What I understood so far about timers in the early frog embryos is that most people think that they involve an mRNA-based mechanism. Maternal mRNAs have a limited stability and disappear suddenly.

Gautier: The mRNA disappears, but one idea would be that there is a protein that inhibits the degradation of this maternal RNA. This is why it is degraded at this time.

Lehner: This suggestion is not sufficient to explain your results with cycloheximide. If cycloheximide is added, the inhibitor should already have been made. In this case the timing can only work if a protein degradation system is switched on on time in the absence of protein synthesis.

Gautier: I agree.

Raff: That is the real mystery. Specific RNA and protein degradations are turned on at precise times, even though there is not RNA or protein synthesis.

Nasmyth: The whole cell cycle can be done without protein synthesis.

Richardson: I wanted to clarify that the apoptosis machinery is present in the early embryo.

Gautier: Yes. One would argue that everything required for apoptosis is present in the egg. If you dissociate the egg and make cell-free extract from the one cell stage embryo, you can in certain conditions have a system to follow apoptosis.

Nurse: Why are checkpoints present in the early frog embryo?

Maller: In some cases I think it is a matter of thresholds. If you add enough DNA to an egg extract, you have a signal sent and heard. If it is a DNA-based signal, you need a certain amount of DNA to send the signal. The amount of DNA required is different for each checkpoint. Only 4N of irradiated DNA (4 nuclei/ μ l) is sufficient for inducing apoptosis at the MBT. Andrew Murray showed that the spindle checkpoint requires a much higher amount of DNA (10 000 nuclei/ μ l) in the system to respond to nocodazole than does the replication checkpoint with aphidicolin (1000 nuclei/ μ l).

Nurse: So you would say there is no biological significance to it; it is simply that because you have so much cytoplasm to DNA, the thing just can't work.

Maller: You need a nuclear:cytoplasmic ratio that can send an adequate signal to be heard. I would speculate that the signal is sent, even when there is only one

nucleus, but it may not be heard by the cell cycle or apoptotic machinery that is present in great abundance.

Nasmyth: There is a similar situation in *Drosophila*, where you also have a high cytoplasm:nucleus ratio. The only problem in *Xenopus* is that the whole cell cycle clock is occurring throughout the entire cytoplasm, whereas *Drosophila* has a certain degree of nuclear autonomy. Even there, these controls are missing to some extent.

Lehner: The spindle checkpoint works well and the DNA replication checkpoint is compromised but present in early *Drosophila* embryos. In addition, Sullivan has proposed that there is a back-up checkpoint mechanism during the syncytial stages (Fogarty et al 1997). If nuclei continue to go through the cell cycle in the presence of unreplicated DNA, they get disposed. The nuclei lose the connection with the centrosome, they fall into the interior of the egg and fail to become cellularized.

Maller: An analogous event occurs in *Xenopus*. Low-level irradiation results in a few damaged cells being removed into the blastocoel, and development proceeds normally without apoptosis.

Nurse: Is there any evidence that checkpoint signals are changing during development in other ways in other systems?

Kubiak: In mouse, there is a weak checkpoint in meiosis for spindle formation. There are some responses where if the spindle is destroyed, the passage from metaphase I to metaphase II is blocked. There is other evidence that there is no checkpoint. I think there is a special checkpoint in meiosis. Starting from the first mitotic division there is no spindle checkpoint.

Nurse: What about replication DNA damage?

Harper: Embryonic stem (ES) cells used to be thought of as not having checkpoints, but recent work has shown that there are γ -irradiation inducible checkpoints in ES cells.

Nurse: Are there changes in checkpoints in other circumstances in development?

Edgar: Imaginal disc cells arrest in G2 when you irradiate them, not G1.

Nurse: I assume that the cells are mainly in G1 when you irradiate them.

Edgar: A lot of them are.

Nasmyth: In budding yeast in meiosis there is G2 arrest, which never occurs in mitotic cells.

Reference

Fogarty P, Campbell SD, Abu-Shumays R et al 1997 The *Drosophila grapes* gene is related to checkpoint gene *chk1/rad27* and is required for late syncytial division. *Curr Biol* 7:418-426

Mre11 Protein Complex Prevents Double-Strand Break Accumulation during Chromosomal DNA Replication

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Summary

Mre11 complex promotes repair of DNA double-strand breaks (DSBs). *Xenopus* Mre11 (X-Mre11) has been cloned, and its role in DNA replication and DNA damage checkpoint studied in cell-free extracts. DSBs stimulate the phosphorylation and 3'-5' exonuclease activity of X-Mre11 complex. This induced phosphorylation is ATM independent. Phosphorylated X-Mre11 is found associated with replicating nuclei. X-Mre11 complex is required to yield normal DNA replication products. Genomic DNA replicated in extracts immunodepleted of X-Mre11 complex accumulates DSBs as demonstrated by TUNEL assay and reactivity to phosphorylated histone H2AX antibodies. In contrast, the ATM-dependent DNA damage checkpoint that blocks DNA replication initiation is X-Mre11 independent. These results strongly suggest that the function of X-Mre11 complex is to repair DSBs that arise during normal DNA replication, thus unraveling a critical link between recombination-dependent repair and DNA replication.

Introduction

Damage to chromosomal DNA induces a complex cellular response designed to delay cell cycle progression until the DNA is repaired (Hensey and Gautier, 1995; Zhou and Elledge, 2000). Surveillance mechanisms monitor the integrity of the genome. Detection of aberrant DNA and chromosome structures coordinately triggers checkpoint pathways that prevent cell cycle progression and activate DNA repair systems.

Response to DNA damage is critical for cell viability. Unchecked DNA damage can lead to mutations, translocations, and abnormal recombination events during

S-phase, and also to chromosome breakage and loss during mitosis. Failure to monitor or to signal damaged DNA is a hallmark of cancer cells (Hartwell and Kastan, 1994). Several cancer-prone syndromes reflect defects in the DNA damage response (Shiloh, 1997). The inherited diseases Ataxia-Telangiectasia (A-T), Ataxia-Telangiectasia like disorder (A-TLD), and Nijmegen Breakage Syndrome (NBS) have similar and overlapping phenotypes, and cells derived from these patients have served as a paradigm for the study of checkpoint pathways (Petrini, 2000). In addition to increased risk for cancer, particularly lymphomas, patients affected with these genetic defects are radiation sensitive. Cells derived from these patients exhibit chromosome fragility and radiosensitivity. They also display radio-resistant DNA Synthesis (RDS). A-T, NBS and A-TLD are caused by mutations in ATM, Nbs1/Nibrin, and Mre11, respectively (Carney et al., 1998; Savitsky et al., 1995; Stewart et al., 1999).

Mre11 forms a tight complex with Rad50 and p95/Xrs2 in *S. cerevisiae* and a similar complex with Rad50 and p95/Nbs1 in humans (Bressan et al., 1999; Dolganov et al., 1996). Biochemical fractionation of human cells indicates that most of Mre11 is in a protein complex that copurifies with Rad50 (Dolganov et al., 1996).

The function of Mre11 is best understood in *S. cerevisiae*, where, along with Rad50, it promotes repair of double-strand chromosome breaks by homologous recombination (HR) and nonhomologous end joining (NHEJ) (Bressan et al., 1999; Haber, 1998; Ogawa et al., 1995). *S. cerevisiae* Mre11 or Rad50 deletion mutants are viable, although they grow slowly (Haber, 1998). *C. elegans* homozygous for an *mre-11* null mutation are viable (Chin and Villeneuve, 2001). In contrast, deletion of *mre11* in mouse or chicken cells is lethal (Yamaguchi-Iwai et al., 1999; Zhao et al., 2000). Deletion of *rad50* or *nbs1* in mouse is also lethal (Luo et al., 1999; Zhu et al., 2001). Why Mre11, Rad50, and Nbs1 are essential in vertebrates in the absence of external DNA damage is unknown. The similarities between the terminal phenotypes of cells lacking Mre11, Rad50, or Nbs1 suggest that these proteins exert their essential role(s) together in a complex. In vitro, yeast and human Mre11/Rad50 complexes act as Mn²⁺-dependent double-stranded DNA 3'-5' exonucleases and single-stranded endonucleases (Moreau et al., 1999; Paull and Gellert, 1998; Trujillo et al., 1998). The nuclease activities of the human complex are enhanced by p95/Nbs1 (Paull and Gellert, 1999). The relationship between these in vitro enzymatic activities and the in vivo DNA repair functions of Mre11/Rad50/Nbs1 remains to be established.

Mre11 complexes form foci at the sites of DNA damage and disperse after repair, consistent with a role in the DNA damage response (Mirzoeva and Petrini, 2001). Of particular pertinence to the work described in this paper, foci containing Mre11 are also detected during S-phase (Mirzoeva and Petrini, 2001). An isoform of histone H2A, H2AX, becomes phosphorylated and colocalizes with these foci following DNA damage (Chen et al., 2000; Paull et al., 2000; Rogakou et al., 1999).

DNA damage induces the phosphorylation of both

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Xenopus Mre11  REDEESSWFLFV|HONHRSKKGPTNF|PEQFDLFLDLY|WGHMECK|ATRNKEQOLFV|SQPGSSVATSLSPGEAAEKKHVGGLR|IKGKHMNMHNK|PHTYR
Human Mre11  KEDENSDFLV|HONHRSKKGPTNF|PEQFDLFLDLY|WGHMECK|ATRNKEQOLFV|SQPGSSVATSLSPGEAAEKKHVGGLR|IKGKHMNMHNK|PHTYR

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Xenopus Mre11  KDSITLINF GKGKIDSKPLLEG TLRVEDLKVYK FGTAEKKNVLSLTERGMOGEAFYDVKEDK DAELEDK DEEVFRRFET
Human Mre11  TGE EINF GKLTLKTP SEGTTRVEDLKVYK FGTAEKKNVLSLTERGMOGEAFYDVKEDK DAELEDK DEEVFRRFET

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Figure 1. X-Mre11 Is Homologous to Human Mre11

The deduced amino acid sequence of *Xenopus* Mre11 was aligned with the sequence of the human Mre11 protein using the Geneworks program (Intellegenetics). Amino acid identities are boxed in yellow.

Mre11 and Nbs1. Mre11 modification occurs in ATM-deficient cells (Dong et al., 1999), whereas phosphorylation of Nbs1 is ATM-dependent. The role of Nbs1 in the DNA damage checkpoint is controversial. Although Nijmegen breakage syndrome cells show no major defects in cell cycle checkpoint (Girard et al., 2000), elimination of the principal ATM phosphorylation site in Nbs1 attenuates the checkpoint (Gatei et al., 2000; Lim et al., 2000; Zhao et al., 2000). Localization of Mre11 to ionizing radiation-induced foci does not require ATM (Mirzoeva and Petrini, 2001).

The role of Mre11 in the DNA damage response is still elusive, in part because it is an essential gene (Xiao and Weaver, 1997; Yamaguchi-Iwai et al., 1999) and because it is involved in several aspects of the response (Haber, 1998). To circumvent these difficulties, we decided to study the functions of Mre11 in cell-free extracts derived from *Xenopus* eggs. These extracts faithfully recapitulate DNA replication and mitosis, as well as the DNA damage checkpoints that regulate these events. We demonstrate that the *Xenopus* homolog of Mre11 (X-Mre11) is phosphorylated during DNA replication or after exposure to DNA carrying double-strand breaks (DSBs). Mre11 complex is essential to produce normal DNA replication products. Depletion of X-Mre11 complex from extracts leads to the dramatic accumulation of DSBs during DNA replication. However, the ATM-dependent DNA damage checkpoint that operates at the onset of S-phase is unaffected by X-Mre11 complex depletion.

Results

Cloning of *Xenopus* Mre11: X-Mre11

Using a PCR strategy with degenerate oligonucleotide primers designed from regions of the Mre11 proteins conserved among yeast, *Drosophila*, and mammals, we amplified a 470 bp DNA fragment that was subsequently used to isolate a full-length clone from a *Xenopus* cDNA library (see Experimental Procedures). The predicted amino acid sequence of X-Mre11 is 711 amino acids.

long. As expected from the similarity among Mre11 proteins from *S. cerevisiae*, *C. elegans*, *D. melanogaster*, and *H. sapiens*, X-Mre11 is highly homologous to the human protein. A protein alignment between the *Xenopus* and human proteins is presented in Figure 1. They are 70% identical over their whole length, and the phosphoesterase/nuclease N-terminal portions of the proteins are the most conserved (83% identity).

Full-length X-Mre11 protein was expressed in *E. coli* and gel purified (see Experimental Procedures). The purified protein was used to generate rabbit polyclonal antibodies. The antiserum recognizes the recombinant protein from *E. coli* lysates (data not shown) and a single polypeptide on Western blots of *Xenopus* egg cytosol (Figure 2A, first lane). When used for immunodepletion, the antibody quantitatively removes X-Mre11 from the extract (Figure 2A, third lane).

X-Mre11 Is a Phosphoprotein

Several proteins involved in the DNA damage response are phosphorylated in response to ionizing radiation. Because radiation generates a variety of aberrant DNA structures, we asked if a defined radiation product, DSBs, could induce X-Mre11 phosphorylation in *Xenopus* interphase extracts. Western blot analysis revealed that a fraction of the X-Mre11 migrated slower on a polyacrylamide gel after exposure to DSBs (Figure 2B, lane 3). This fraction represented phosphorylated X-Mre11, because the band was eliminated by phosphatase treatment. Interestingly, phosphatase treatment converted X-Mre11 from control or DSB-treated extracts into a polypeptide that migrated faster than X-Mre11 (Figure 2B, lanes 2 and 4).

To confirm that X-Mre11 is a phosphoprotein and that its phosphorylation increases following incubation with DSBs, X-Mre11 was immunoprecipitated from γ -³²P-ATP-labeled control extracts or extracts incubated with DSBs (Figure 2B, right panel). No ³²P-labeled proteins were precipitated by preimmune serum (Figure 2B, PRE). A ³²P-labeled polypeptide was immunoprecipitated with X-Mre11 antibodies from extracts treated with

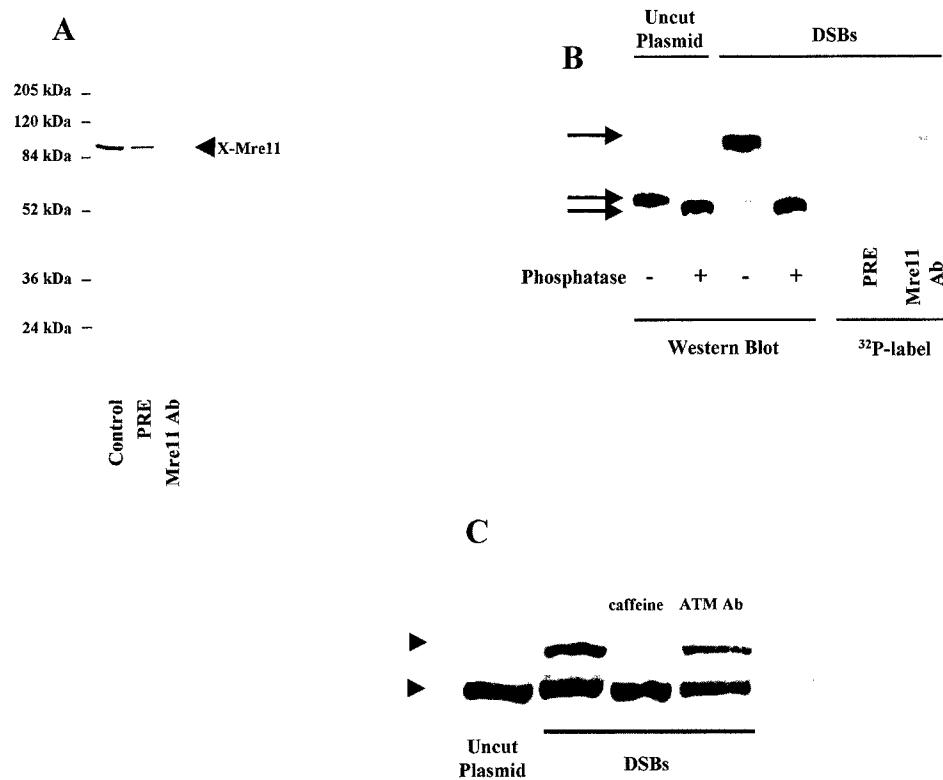


Figure 2. X-Mre11 Is Phosphorylated in a DSBs-Dependent, ATM-Independent Manner

(A) Untreated interphase extract (Control), extract incubated with protein A beads bound to preimmune serum (PRE), and extract incubated with protein A beads bound to anti-X-Mre11 antibodies (X-Mre11 Ab) were probed with a polyclonal serum specific for X-Mre11 protein. (B) Left panel. X-Mre11 was immunoprecipitated from extracts containing either uncut plasmid (lanes 1 and 2) or DNA containing double strand breaks (DSBs) (lanes 3 and 4; see Experimental Procedures). In lanes 2 and 4, the proteins were treated with λ phosphatase (+). Arrows on the left indicate X-Mre11 isoforms. Right panel. *Xenopus* interphase extracts were labeled with γ^{32} P-ATP, immunoprecipitated with preimmune (PRE) or anti-X-Mre11 antibodies (Mre11 Ab) in presence of DSBs and processed for autoradiography following SDS-PAGE. (C) *Xenopus* extracts were subjected to PAGE followed by Western blotting with X-Mre11-specific antibody. Interphase extracts were either treated with circular plasmid (uncut plasmid) or with DSBs. Extracts treated with DSBs were incubated with 5 mM caffeine (caffeine) or anti-X-ATM antibodies (ATM Ab), as indicated. Arrows on the left indicate X-Mre11 isoforms.

DSBs (Figure 2B, Mre11 Ab). By aligning the 32 P-labeled proteins with the Western blot probed with X-Mre11 antibodies (Figure 2B, left panel), we determined that the 32 P-labeled polypeptide corresponds to the hyperphosphorylated X-Mre11 that arose in response to DSBs.

Nbs1/p95 forms a tight complex with X-Mre11 in mammalian cells and is phosphorylated following DNA damage via a pathway that requires ATM protein kinase. We asked if X-Mre11 phosphorylation induced by DSBs was likewise ATM-dependent. To inhibit ATM, we used a specific anti-X-ATM antibody (Robertson et al., 1999) at a concentration that completely abrogates the ATM-dependent DNA damage checkpoint (Costanzo et al., 2000). The antibody failed to block induced X-Mre11 phosphorylation (Figure 2C, lane 4). In contrast, caffeine, which inhibits ATM, ATR as well as the Chk family of protein kinases (Sarkaria et al., 1999), completely abolished induced X-Mre11 phosphorylation (Figure 2C, lane 3). The caffeine-sensitive protein kinase responsible for the DSB-induced X-Mre11 modification remains to be identified, although it is clear from these experiments that it is not ATM.

DSB-Induced Phosphorylation Stimulates X-Mre11-Associated Exonuclease

We then asked if phosphorylation affected the 3'-5' exonuclease activity of X-Mre11 complex. This enzymatic activity requires the association of Mre11 with Rad50 and is enhanced by Nbs1/p95 (Paull and Gellert, 1998; Paull and Gellert, 1999).

X-Mre11 complexes were immunoprecipitated under native conditions and assayed for 3'-5' exonuclease activity using different oligonucleotide substrates. End-labeling or internal labeling of the substrates gave identical results (data not shown). As reported for the recombinant human complex, X-Mre11 complex digested double-strand oligonucleotides with blunt or 5' overhanging ends, whereas double-strand oligonucleotides with 3' overhangs were not processed (data not shown). X-Mre11 complex precipitated from control extracts was active (Figure 3, column 1). Preincubating the extracts with DSBs, however, increased the enzymatic activity of the X-Mre11 complex by 70% as established by three independent experiments (Figure 3, column 2).

DSB stimulation of exonuclease activity correlated with the phosphorylation status of X-Mre11. Caffeine,

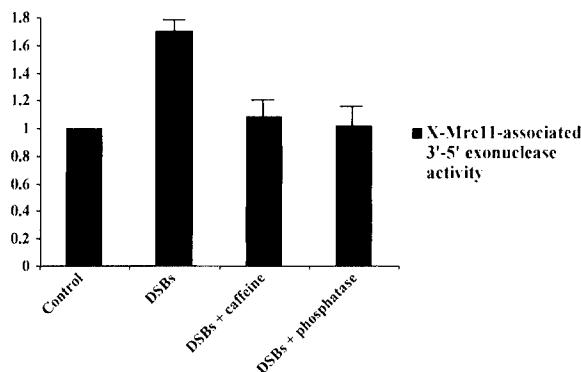


Figure 3. X-Mre11 Exonuclease Activity Is Regulated by DSB-Induced Phosphorylation

X-Mre11 was immunoprecipitated from interphase extracts and incubated with labeled oligonucleotide substrate (see Experimental Procedures) for 60 min. Mre11 exonuclease activity was measured by the amount of degraded substrate. Mre11 exonuclease activity is expressed in arbitrary units; uninduced activity = 1. The first column represents the uninduced activity of X-Mre11 precipitated from an extract treated with circular plasmid (Control). Columns 2 and 3 represent the activity of X-Mre11 precipitated from an extract treated with DSBs or DSBs + caffeine (DSBs + caffeine), respectively. Column 4 represents the activity of X-Mre11 precipitated from a DSB-treated extract that was subsequently treated with λ phosphatase. The data presented are from three independent experiments.

which inhibits X-Mre11 phosphorylation (Figure 2C, lane 3), abolished stimulation (Figure 3, column 3). Moreover, phosphatase treatment of stimulated X-Mre11 complex reduced exonuclease activity to basal levels (Figure 3, column 4). These results establish that DSBs stimulate X-Mre11 complex exonuclease via a pathway that entails phosphorylation of X-Mre11 and/or an associated protein.

X-Mre11 Binds to Chromatin and Is Activated during DNA Replication

To explore further the physiological significance of X-Mre11 phosphorylation, we asked if X-Mre11 was modified during chromosomal DNA replication in the absence of added DSBs. Demembranated *Xenopus* sperm nuclei were incubated in extracts to allow chromatin assembly and DNA replication. The nuclei were then purified, and chromatin-associated X-Mre11 was demonstrated by Western blot. In the absence of incubation, no X-Mre11 was detected in the nuclear fraction (Figure 4A, lane 2). X-Mre11 became associated with the nuclear fraction during replication (Figure 4A, lanes 3 and 4) and remained in association after replication was completed (Figure 4B, lanes 1–3).

To trap phosphorylated intermediates, we added tautomycin, a phosphatase inhibitor, 30 min following addition of nuclei. Under these conditions, at least two hyperphosphorylated forms of X-Mre11 appeared in the nuclear fraction (Figure 4A, lanes 5 and 6). In contrast, cytoplasmic X-Mre11 isolated from the tautomycin-treated extracts at 90 min was not hyperphosphorylated. Thus, this modification of X-Mre11 depends upon its nuclear localization (Figure 4A, lane 1). It also requires DNA replication. When DNA replication was blocked by

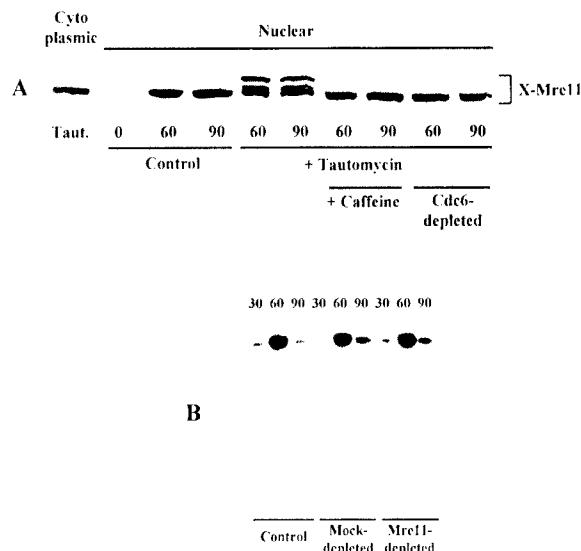


Figure 4. X-Mre11 Is Phosphorylated during DNA Replication, and Is Not Required for DNA Replication Initiation or Elongation

(A) Cytoplasmic and nuclear fractions (see Experimental Procedures) were analyzed by Western blot with anti-X-Mre11 antibodies under the following conditions. Cytoplasm was purified after 90 min incubation with nuclei in the presence of tautomycin (lane 1). Nuclei were incubated for 0, 60, and 90 min in untreated interphase extracts (lanes 2–4). Nuclei were incubated for 60 and 90 min in extracts to which tautomycin was added at 30 min following addition of nuclei (lanes 5 and 6). Nuclei were incubated for 60 and 90 min in extracts treated with tautomycin and caffeine (lanes 7 and 8). Nuclei were incubated for 60 and 90 min in cdc6-depleted extracts in the presence of tautomycin (lanes 9 and 10).

(B) Genomic DNA replication was monitored by 30 min pulses of $\alpha^{32}\text{P}$ -ATP. Incorporation into chromatin incubated with untreated extract (Control), extract incubated with protein A beads bound to preimmune serum (Mock-depleted), or extract incubated with protein A beads bound to anti-X-Mre11 antibodies (Mre11 complex-depleted).

depleting extracts of Cdc6, X-Mre11 appeared in the nuclear fraction, but was not hyperphosphorylated (Figure 4B, lanes 9 and 10).

We then asked if the modification of X-Mre11 during DNA synthesis might be due to DSB formation in the replicating DNA. Consistent with this idea, we found that replication-dependent phosphorylation of X-Mre11, like DSB-induced phosphorylation, was sensitive to caffeine (Figure 4A, lanes 7 and 8). Taken together, these results indicate that X-Mre11 associates with chromatin and is phosphorylated, possibly in a DSB-dependent manner, during DNA replication.

X-Mre11 Prevents the Accumulation of DSBs during DNA Replication

A role for Mre11 in DNA replication has been suggested (Petrini, 2000). We therefore asked if X-Mre11 complex depletion affected DNA synthesis. Genomic DNA was pulse-labeled with radioactively labeled deoxynucleotides at various times after addition of nuclei. Control, mock-depleted and extracts quantitatively depleted of X-Mre11 complex, were compared. The kinetics and the levels of incorporation of radionucleotides into genomic DNA, separated by agarose gel electrophoresis, were

identical in the three extracts (Figure 4B). X-Mre11 is, therefore, required neither for DNA replication initiation nor elongation.

We then performed experiments to demonstrate that, in fact, DSBs arise during normal DNA replication, and that, furthermore, activated X-Mre11 complex plays a role in their repair. We designed a TUNEL-based assay to monitor the accumulation of DSBs during DNA replication in the presence or absence of X-Mre11 complex (see Experimental Procedures). Nuclei were replicated in control or in X-Mre11 complex-depleted extracts. At 100 min after incubation, the postreplicative genomic DNA was extracted and end-labeled using terminal transferase (TdT) and radioactive deoxynucleotides (Figures 5A–5C).

Demembranated sperm nuclei (Figure 5A, columns 1 and 2), postreplicative nuclei replicated in a control extract (Figure 5A, columns 3 and 4), or in mock-depleted extracts (Figure 5A, columns 5 and 6) were not significantly end-labeled with TdT. In contrast, nuclei replicated in extracts depleted of X-Mre11 complex were extensively end-labeled (Figure 5B, column 8). Labeling was TdT-dependent (Figure 5A, column 7) and was resistant to aphidicolin, indicating it was not due to contaminating DNA polymerase (Figure 5A, column 10). To establish that the increase in end-labeling was due to the removal of X-Mre11 complex, human Mre11/Rad50/Nbs1 complex, purified from baculovirus-infected cells (Paull and Gellert, 1999), was added to depleted extracts prior to replication. The added complex reduced end-labeling to control levels (Figure 5B, columns 1 and 2). Depletion of X-Mre11 complex does not prevent TdT access to DSBs. Mre11/Rad50/Nbs1 complex, added to the in vitro reaction on postreplicative nuclei prior to TdT, did not inhibit TdT labeling (Figure 5A, column 9).

Nuclei isolated during DNA replication in control extracts were not end-labeled by TdT (Figure 5B, columns 3–5). Thus, single-stranded DNA fragments generated during DNA replication (Okazaki fragments) are not a substrate for TdT under our experimental conditions. Inhibiting DNA replication with 6-dimethylaminopurine (6-DMAP, a protein kinase inhibitor), by depletion of Cdc6 or with actinomycin D (a primase inhibitor), blocked end-labeling in the depleted extracts (Figure 5B, lanes 6–8). These data demonstrate that MRE11-complex prevents the accumulation of DSBs in replicating genomic DNA.

Inactivation of *mre11* in chicken cells leads to rapid apoptosis (Yamaguchi-Iwai et al., 1999). We wished to assess whether the accumulation of DSBs in depleted extracts was a consequence of apoptosis. First, we showed that ZVAD, a potent peptide inhibitor of caspase, did not inhibit end-labeling in depleted extracts (Figure 5C, compare columns 1 and 2). We next demonstrated that truncated PARP (t-PARP), a substrate for caspase-3 cleavage (Hensey and Gautier, 1997) was stable in control, mock-depleted, or depleted extracts (Figure 5D). As expected, t-PARP was completely cleaved following addition of cytochrome C. Finally, we quantified the concentration of DSBs following induction of apoptosis. Nuclear end-labeling in extracts treated with cytochrome C was 7-fold higher than in depleted extracts (Figure 5C, column 4). This establishes

that the accumulation of DSBs in nuclei replicated in the absence of X-Mre11 complex is not a consequence of apoptosis.

We confirmed the accumulation of DSBs in depleted extracts with an assay unrelated to TdT end-labeling. Histone H2AX, phosphorylated at serine residue 139 (phospho-H2AX), is found in association with DSBs in chromatin (Rogakou et al., 1998). We obtained an antibody against mammalian phospho-H2AX and demonstrated that it recognized the *Xenopus* homolog. Sperm chromatin was partially digested with restriction endonuclease NotI to create DSBs. The chromatin was incubated for 1 hr in interphase extract, then purified and probed with the phospho-H2AX antibody. Phospho-H2AX was detected in the NotI-treated sample (Figure 5E, lane 1), whereas undigested chromatin showed no signal (Figure 5E, lane 2). We then isolated nuclei that were replicated in mock-depleted or depleted extracts. As is clear from Figure 5D, left panel (compare lanes 3 and 4), postreplicative nuclei isolated from extracts depleted of Mre11 complex contained phospho-H2AX, and therefore, DSBs. The concentration of DSBs induced by NotI as assessed from the TUNEL and the immunological assays were comparable (Figure 5C, lanes 1 and 3). Phosphorylation of histone H2AX in depleted extracts was inhibited in a dose-dependent manner by human Mre11/Rad50/Nbs1 complex added prior to DNA replication (Figure 5E, right panel, lanes 1–4). Phosphorylation of histone H2AX was dependent upon DNA replication; Cdc6 depletion entirely eliminated the phospho-H2AX signal (Figure 5E, right panel, lane 5).

Taken together, these data establish that DSBs arise during genomic DNA replication and that X-Mre11 protein complex blocks their accumulation.

X-Mre11 Is Not Required for the DNA Damage Checkpoint that Inhibits S-Phase Entry

In mammalian cells, Mre11 associates with Rad50 and Nbs1/p95. Phosphorylation of Nbs1/p95 by ATM following DNA damage is important for the DNA damage-induced S-phase checkpoint (Gatei et al., 2000; Lim et al., 2000; Wu et al., 2000; Zhao et al., 2000). We have described an in vitro system based upon partially purified *Xenopus* extracts that recapitulates the ATM-dependent DNA damage checkpoint (Costanzo et al., 2000). Using this system, we asked if X-Mre11 plays a role in the checkpoint. Initiation of DNA replication is monitored after mixing 2 fractions (M and B) prepared from untreated extracts or extracts treated with DSBs (M* and B*). Both fractions are required to initiate replication of chromosomal DNA.

A DSB-induced checkpoint, as indicated by the absence of DNA replication, was seen in mixtures containing fractions M* and B* (compare lane 3 and 5, Figure 6). M* and B* fractions prepared from X-Mre11 complex depleted extracts (Figure 2A) likewise failed to support DNA replication, indicating a functional checkpoint (Figure 6, lane 6). As previously described, DNA replicated efficiently in the presence of fractions derived from ATM-inhibited extracts treated with DSBs (Figure 6 lane 7 and Costanzo et al., 2000). This establishes that X-Mre11 complex is not required for the ATM-dependent checkpoint that prevents initiation of DNA synthesis following DNA damage.

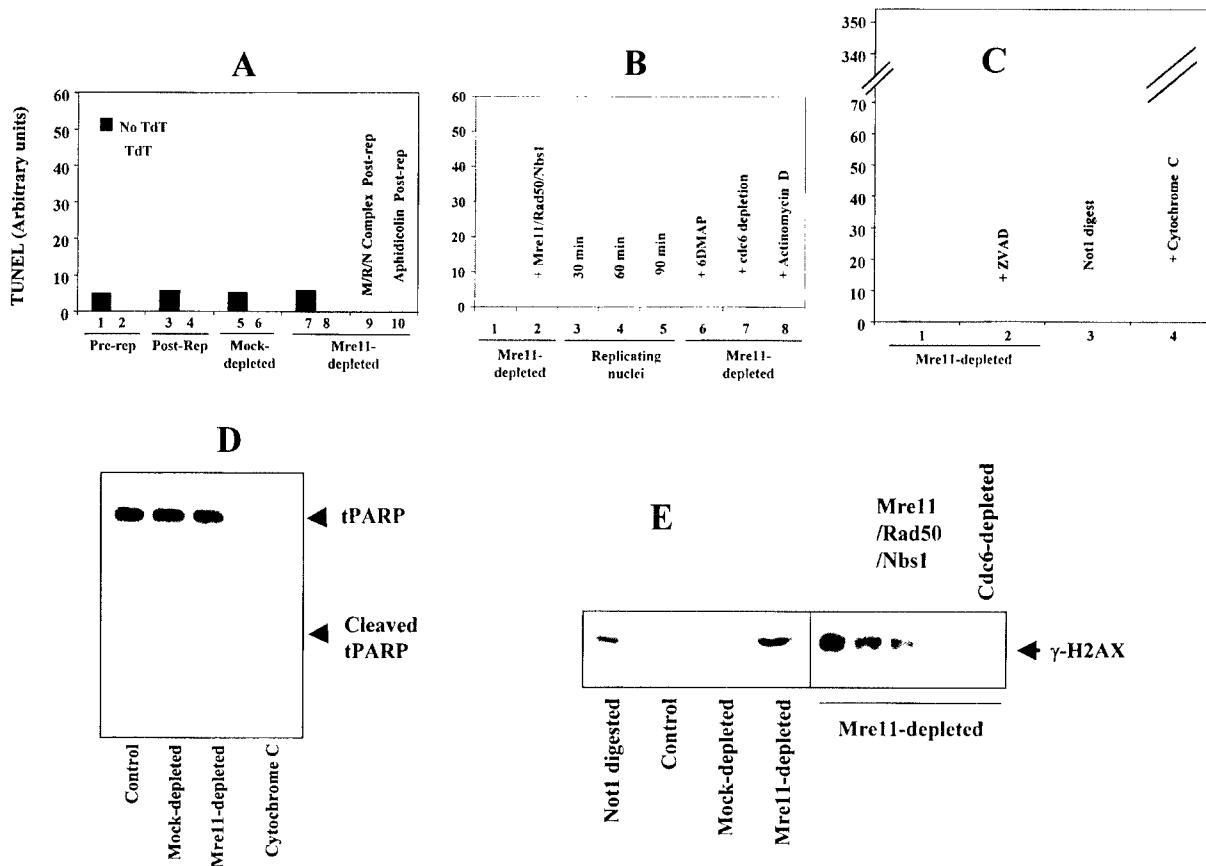


Figure 5. X-Mre11 Prevents the Accumulation of DSBs that Arise during DNA Replication

(A–C) DSBs were detected by TUNEL assay measuring incorporation of $\gamma^{32}\text{P}$ -dGTP into genomic DNA.

(A) Labeling of prereplicative nuclei (columns 1 and 2), postreplicative nuclei isolated from a control extract (columns 3 and 4), a mock-depleted extract (columns 5 and 6), and a X-Mre11 complex-depleted extract (columns 7 and 8). Controls were incubated without terminal transferase (No TdT, columns 1, 3, 5, and 7). Nuclei replicated in X-Mre11 depleted extracts were purified and incubated with TdT in the presence of 500 nM Mre11/Rad50/Nbs1 complex (column 9). Labeling of postreplicative nuclei replicated in X-Mre11 complex-depleted extracts in the presence of aphidicolin (column 10).

(B) Labeling of nuclei replicated in a X-Mre11 complex-depleted extracts (column 1) and in a X-Mre11 complex-depleted extract supplemented with 500 nM human Mre11/Rad50/Nbs1 (Paull and Gellert, 1999) recombinant complex prior to replication (column 2). Labeling of nuclei during replication in untreated extracts at 30 min (column 3), 60 min (column 4), and 90 min (column 5). Labeling of nuclei incubated in a X-Mre11 complex-depleted extracts in which DNA replication was inhibited by 6DMAP (column 6), by Cdc6-depletion (column 7), or by addition of actinomycin D (column 8).

(C) Labeling of nuclei replicated in X-Mre11 complex-depleted extracts (column 1) and in a X-Mre11 complex-depleted extract in the presence of ZVAD. Labeling of nuclei partially digested with Not1 (column 3). Labeling of nuclei incubated in extract in the presence of cytochrome C (column 4).

(D) Cleavage of radiolabeled tPARP in various extracts. tPARP cleavage in normal extracts (Control), extracts treated with preimmune serum (PRE), X-Mre11-depleted extracts (Depleted), and in Cytochrome C-treated extracts (Cyto C).

(E) Chromatin fractions were analyzed by Western blot with γ -H2AX antibodies under the following conditions: Left panel. Chromatin partially digested with Not1 (lane 1). Chromatin extracted from nuclei replicated in a control extract (lane 2), a mock-depleted extract (lane 3), and a X-Mre11 complex-depleted extract (lane 4). Right panel. Chromatin extracted from nuclei replicated in a X-Mre11 complex-depleted extract (lane 1) and in a X-Mre11 complex-depleted extracts supplemented with increasing amounts of human Mre11/Rad50/Nbs1 recombinant complex (lanes 2: 125 nM, 3: 250 nM and 4: 500 nM). Chromatin extracted from nuclei incubated in Cdc6-depleted extract (lane 5).

Discussion

Regulation of Mre11 Activity

We isolated the *Xenopus* homolog of Mre11 (X-Mre11) and showed that it is similar to other Mre11 family members, from yeast to human (Haber, 1998; Petrini et al., 1995; Tavassoli et al., 1995). The Mre11 phosphoesterase motifs, which are important for the catalytic activity of the protein (Bressan et al., 1998; Haber, 1998; Moreau et al., 1999), are particularly well conserved.

Our data suggests that the molecular architecture of

the complexes formed with X-Mre11 is also conserved. When X-Mre11 immunoprecipitates were probed with an antibody against mouse Rad50, we observed a 150 kDa crossreacting protein similar in molecular weight to mouse Rad50 (data not shown). This observation suggests that, like its mammalian counterpart, X-Mre11 is present in a complex with other proteins, including X-Rad50. Therefore, immunodepletion removes an X-Mre11 complex.

The *Xenopus* and human Mre11 complexes (Paull and Gellert, 1998) display 3'-5' exonuclease activity with

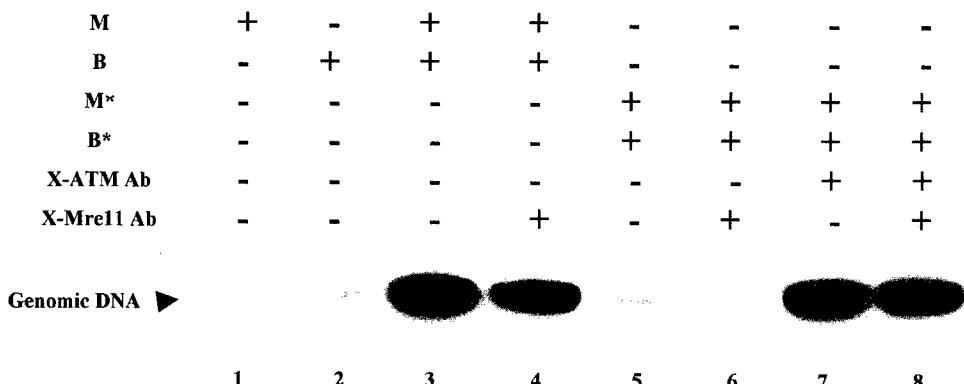


Figure 6. X-Mre11 Is Not Required for the DSB-Induced, ATM-Dependent Checkpoint

DNA replication was assayed in reconstituted extracts consisting of 6-DMAP-treated chromatin and fractions M and B prepared from different sources as indicated. Fractions M and B are derived from extracts treated with circular plasmid DNA. Fractions M* and B* are derived from extracts treated with DSBs. M and B (lanes 3 and 4), as well as M* and B* fractions (lanes 5–8), were prepared from extracts in which X-ATM was inhibited with specific antibodies (X-ATM, lanes 7–8) or extracts in which X-Mre11 complex was depleted (X-Mre11 Ab, lanes 4, 6, and 8), as indicated.

similar substrate specificity (data not shown). Both exonucleases process blunt or 5' overhanging DSBs, but cannot degrade DSBs with 3' overhangs.

The X-Mre11-associated exonuclease is stimulated by addition of DSBs to the extracts in the absence of any other aberrant DNA structures. Importantly, stimulation is accompanied by and dependent upon phosphorylation of X-Mre11 complex. Caffeine that prevents X-Mre11 phosphorylation, or phosphatase treatment of activated X-Mre11 complex, reduce exonuclease activity to basal levels. Mammalian Mre11 was shown to be phosphorylated following ionizing radiation, although, in this case, the precise nature of the inducing factor(s) is unknown (Dong et al., 1999). In contrast to the irradiation-induced phosphorylation of Nbs1, X-Mre11 phosphorylation induced by DSBs is not dependent on X-ATM. Thus, X-Mre11 phosphorylation occurs in the presence of specific antibodies against X-ATM (Robertson et al., 1999). This agrees with the observation that Mre11 phosphorylation and focus formation is normal in DNA-damaged A-T cells (Dong et al., 1999; Mirzoeva and Petrini, 2001). The kinase responsible for X-Mre11 phosphorylation is not known. However, the reaction is sensitive to 5 mM caffeine. This pattern of inhibition is similar to the mammalian ATM-related (ATR) kinase.

Finally, we showed that X-Mre11 is phosphorylated in control extracts. Addition of DSBs induces phosphorylation at additional sites, as evidenced by a shift in apparent molecular weight on SDS-PAGE gels

DNA DSBs Are Generated during DNA Replication

Although DSBs are known to arise during DNA replication in bacteria, experimental evidence for DNA breakage during eukaryotic replication has not been reported (Cox et al., 2000; Seigneur et al., 1998; Seigneur et al., 2000).

Our data show that DSBs are generated during the normal course of DNA replication in *Xenopus* egg extracts. First, we find that only the nuclear fraction of Mre11 is hyperphosphorylated during replication. Recall

that DSBs induce X-Mre11 phosphorylation, which, like replication-induced phosphorylation, is sensitive to caffeine. Second, when X-Mre11 complex is depleted from the extracts, we observe a dramatic accumulation of DSBs as demonstrated by two different assays for DSBs. Finally, accumulation of DSBs is strictly dependent on DNA replication. We propose that these DSBs are normally very transient and are rapidly removed by activated X-Mre11 complex.

Comparison between TUNEL labeling of postreplicative nuclei in depleted extracts with that of chromatin digested with NotI indicates that each chromosome accumulates, on the average, 5–10 DSBs (data not shown). It is possible that additional breaks occur during replication that are eliminated by alternative repair pathways. The accumulation of DSBs during replication in X-Mre11 complex-depleted extracts is consistent with the effects of depletion on the morphology of condensed chromosomes during mitosis. When postreplicative nuclei were driven into mitosis by addition of metaphase-arrested *Xenopus* extract, normal chromosomes and metaphase spindles formed in nuclei replicated in control extracts whereas nuclei replicated in depleted extracts showed fragmented chromosomes and assembled grossly abnormal spindles (data not shown).

In theory, DSBs could be generated in several ways during replication. They could arise if a replication fork passed through a nicked DNA template or from failure to repair DNA adducts induced by DNA modifying agents. Finally, Holliday junctions formed at stalled replication forks could produce DSBs (Scully et al., 2000; Seigneur et al., 1998; Zou and Rothstein, 1997). Aberrant DNA structures that lead to collapsed replication forks are known to occur at palindromic DNA sequences. Hypothetically, these forks will restart if they are in close proximity to a replication origin, or if the replication block is resolved by Sister Chromatid Exchange (SCE). SCE requires the activity of Mre11, BRCA1, Nbs1, and Rad50 (Scully et al., 2000). Inactivation of any of these genes inhibits recombination-dependent SCE repair and generates DSBs.

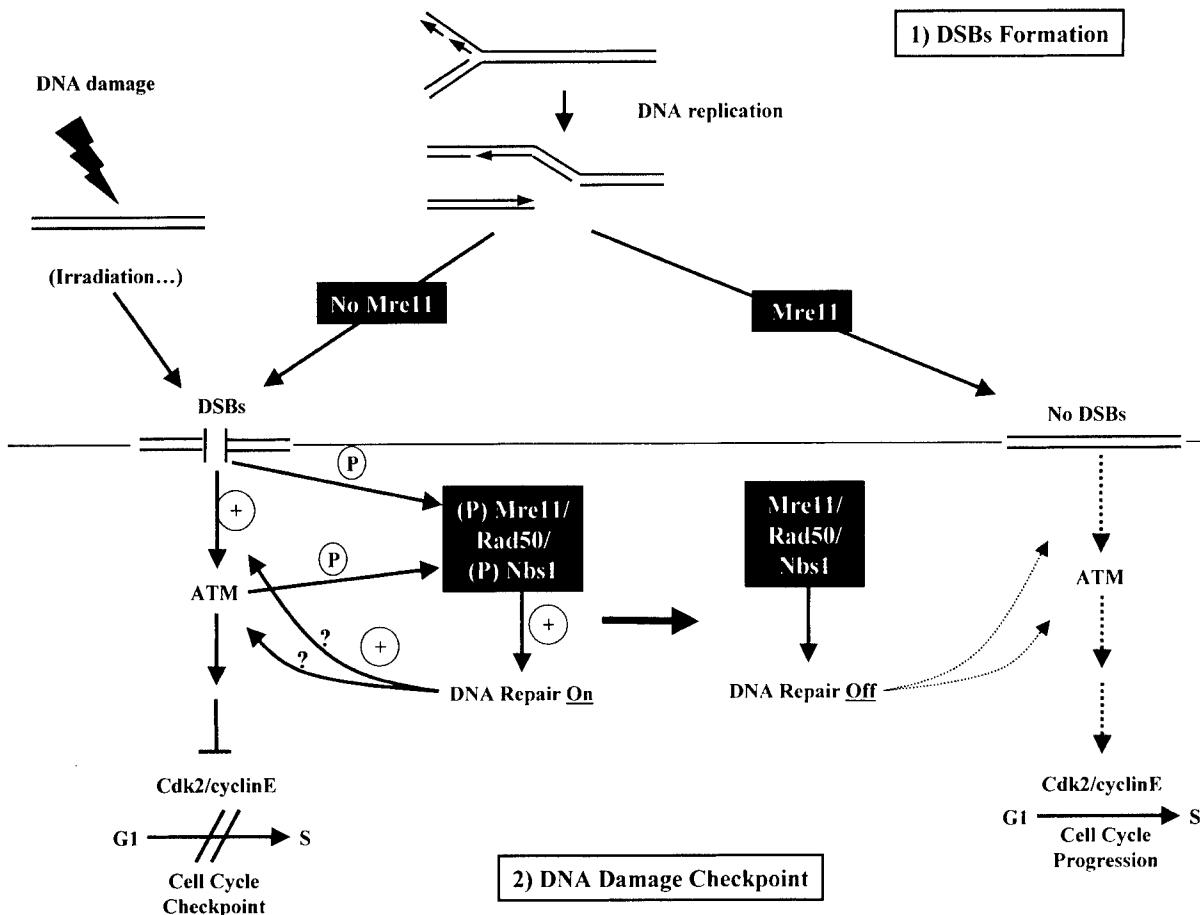


Figure 7. Our Preferred Model for Mre11 Function in DNA Synthesis and the Response to DNA Damage

(1) Top half: Mre11 prevents the accumulation of DSBs during DNA replication. DNA DSBs can arise following external DNA damage or as a consequence of DNA replication.

(2) Bottom half: proposed role for the Mre11/Rad50/Nbs1 complex in the maintenance of the DNA damage checkpoint.

DSBs activate the ATM-dependent checkpoint pathway leading to cdk2 inactivation and inhibition of DNA replication initiation (Costanzo et al., 2000). DSBs also trigger the activation of the Mre11/Rad50/Nbs1 complex through both ATM-dependent and independent phosphorylation. The Mre11/Rad50/Nbs1 complex is essential for the repair of DSBs. We hypothesize that Mre11-driven repair reinforces the ATM-dependent checkpoint (possibly through Nbs1 function) and maintains this checkpoint signaling active during repair. After DSB repair, Mre11 complex is no longer hyperphosphorylated; DNA repair and the ATM-dependent checkpoint are downregulated.

The Essential Function(s) of Mre11

Inactivation of any one of the three components of the Mre11/Rad50/Nbs1 complex in mouse or in chicken cells abolishes normal cell proliferation (Luo et al., 1999; Xiao and Weaver, 1997; Yamaguchi-Iwai et al., 1999; Zhu et al., 2001). Therefore, Mre11 complex is essential for cell viability even in the absence of externally induced DNA damage. It has been difficult to determine the essential role of Mre11 complex because inactivation of any one of the three genes induces a terminal apoptotic-like phenotype. Depletion of X-Mre11 complex from *Xenopus* extracts, however, did not trigger apoptosis, presumably because of the small number of DSBs generated. By extension, our data suggest that in the absence of Mre11 complex in chicken or mouse cells, cell death occurs as a secondary consequence of the loss of genomic integrity. The phenotype that we observe in *Xenopus* is reminiscent of ATR inactivation in mouse, which generates chromosome breaks unaccompanied by caspase activation (Brown and Baltimore, 2000). We

propose, therefore, that one essential function of Mre11/Rad50/Nbs1 complex in chicken or mouse cells is to prevent DNA replication-dependent DSB formation. Interestingly, Mre11-containing complexes accumulate at stalled DNA replication forks cells from the xeroderma pigmentosum variant (XPV) (Limoli et al., 2000). Based upon its established role in DNA repair, we propose that Mre11 complex repairs DSBs as they arise during replication (Figure 7). A fraction of X-Mre11 is nuclear and is hyperphosphorylated during replication. Phosphorylation enhances the nuclease activity of X-Mre11 complex, an activity that is likely to play a role in DSB repair.

mre11 is not essential in *C. elegans* or in *S. cerevisiae* (Chin and Villeneuve, 2001; Ogawa et al., 1995). This might reflect differences in the way yeast and higher eukaryotic chromosomes are replicated, or the presence of redundant systems that repair DSBs. Alternatively, DSBs might arise more frequently in larger and more complex genomes containing repetitive DNA sequences.

We have unraveled a critical link between recombination-dependent DNA repair and DNA replication by demonstrating the requirement for Mre11 complex during DNA replication in the absence of induced DNA damage.

Mre11 and the DNA Damage Checkpoint

Cells from A-TLD or NBS patients, which harbor hypomorphic mutations in *mre11* and *nbs1*, respectively, exhibit radio-resistant DNA synthesis (RDS) (Petrini, 2000). The RDS phenotype, found also in cells from A-T patients (Carney et al., 1998; Stewart et al., 1999), possibly reflects a defect in one or several DNA damage checkpoint(s) operating at the time of initiation of or during DNA replication. It is not clear if *Nbs1* is involved in the DNA damage checkpoint. Although NBS cells show no major defects in cell cycle checkpoint (Girard et al., 2000), phosphorylation of *Nbs1* is important for the G1 to S DNA damage checkpoint (Gatei et al., 2000; Lim et al., 2000; Zhao et al., 2000).

In contrast, we have shown that the DSB-induced, ATM-dependent checkpoint that down-regulates cdk2 and prevents origin activation and firing (Costanzo et al., 2000) is not dependent on Mre11. This is similar to *C. elegans*, where *mre11* mutants undergo the DNA damage checkpoint that induces apoptosis (Chin and Villeneuve, 2001).

First, it is possible that *Nbs1* complexes that lack Mre11 might participate in the mammalian DNA damage checkpoint, whereas complexes that contain both proteins would function in DSB repair. In such a model, depletion of Mre11 would leave *Nbs1* and the checkpoint intact but block repair of DSBs. Evidence for such multiple protein complexes awaits the isolation and characterization of the *Nbs1* *Xenopus* homolog.

Alternatively, *Nbs1* in complex with Mre11 might be required not for the initiation but for the maintenance of the checkpoint. In *Xenopus*, initiation of DNA replication is synchronous, and the rapid ATM-dependent checkpoint activation does not require Mre11/Rad50/*Nbs1* complex. In mammals, where replication initiation is asynchronous, *Nbs1*, and possibly Mre11/Rad50/*Nbs1* complex, might be needed at a later stage during S-phase. *Nbs1* might promote a feedback mechanism that reinforces the ATM-dependent checkpoint until repair is completed (Figure 7). When subjected to irreparable DNA damage, *S. cerevisiae* first activates a DNA damage checkpoint, then resumes cell cycle progression: a phenomenon called adaptation. Mutants defective in adaptation have been isolated that remain arrested in cell cycle after DNA damage. These mutants resume cell cycle progression when either Mre11 or Rad50 are inactivated (Lee et al., 1998), consistent with the idea that Mre11 complex maintains rather than activates the DNA damage checkpoint. This hypothesis is attractive, because it reconciles genetic data with other experimental analysis in different species.

Experimental Procedures

Cloning of the *Xenopus* Homolog of Mre11

To isolate X-Mre11 cDNAs, we synthesized degenerate primers that correspond to coding sequences for amino acids highly conserved among the Mre11 proteins from human, mouse, *Caenorhabditis elegans*, *Saccharomyces cerevisiae*, and *Schizosaccharomyces*

pombe. The primers F3 (5'-GAY YTB TTY CAY GAR AAY AAR CC) and R4 (5'-TGD ATN ACR AAN ARR TTR AAC CA) represent codons 60–67 and 210–217 of the human sequence. First strand cDNA was synthesized from *Xenopus laevis* oocyte mRNA using a polyT primer and Super Script II reverse transcriptase (Gibco BRL). Amplification with R3 and F4 PCR primers was carried out for 30 cycles of 30 s at 95°C, 40 s at 54°C, and 60 s at 72°C. A fragment of the expected size (470 bp) was obtained and was cloned into pBluescript KS+. The sequences of the inserts in several clones were determined and shown to correspond to X-Mre11. The same PCR fragment was labeled by random priming and used to screen a *X. laevis* tadpole head cDNA library in the λZAP vector (Hemmati-Bivanlou et al., 1991). Plaque lifts were prepared and hybridized as previously described (Bibikova et al., 1998), and phagemids carrying the inserts were excised according to the supplier's protocol (Stratagene). Two clones containing the complete coding sequence, plus 5' and 3' untranslated regions were identified and sequenced (GenBank Accession # AF134569).

Antibody Production

The coding region of X-Mre11 was amplified from the phagemid using Pfu DNA polymerase (Stratagene) and the primers 5'-CAG CCG GCA CAT ATG AGT TCT TC and 5'-TAA AAC GGA TCC TAT TTA TCT ACG GCC. The former incorporates the ATG codon into an NdeI site at the 5'-end of the cDNA, and the latter puts a BamHI site beyond the termination codon. The resulting PCR product was cloned between the NdeI and BamHI sites of pET16b (Novagen); this links X-Mre11 to an N-terminal His₁₀ tag. Upon transformation into *E. coli* BLR (DE3) and induction with IPTG, this construct led to the production of substantial amounts of insoluble X-Mre11 protein.

The pellet from lysed bacteria was dissolved in 6 M urea, and the His-tagged X-Mre11 was enriched by binding to and elution from a His-bind column (Novagen). The protein was subjected to SDS-PAGE and negatively stained with CuCl₂. Gel slices containing X-Mre11 were excised and sent to Covance Research Products Inc. (P.O. Box 7200, Denver, PA 17517) where rabbit antibodies were raised using standard protocols. The resulting antiserum and pre-immune serum were used for Western blots at 1:2000 dilutions and for immunoprecipitations as described later.

X-Mre11 Detection

Xenopus interphase extracts were prepared according to Murray (1991). Extracts were treated with 10 ng/μl of circular pBR322 or with 10 ng/μl of pBR322 digested with HaeIII and with 5 mM caffeine at 20°C. Extracts were electrophoresed on 10% SDS-PAGE and blotted with X-Mre11 specific antiserum. Analysis of nuclear X-Mre11 was performed in presence of 3 μM tautomycin. Fifty microliters of extract containing 8000 nuclei/μl were diluted in nuclei isolation buffer (100 mM KCl, 25 mM HEPES (pH 7.8), 2.5 mM MgCl₂, and 0.01% Triton X-100) and purified through a 30% sucrose cushion. Samples were spun for 15 min at 6000 × g at 4°C; the pellet were washed once with the same buffer and processed for electrophoresis.

X-Mre11 Activity

5'-Labeled Oligonucleotide Templates

The Tp74 oligo described by Paull and Gellert (1998) was 5'-labeled with γ ³²P-ATP by using T4 polynucleotide kinase according to standard procedures. Unincorporated nucleotides were removed by separation through a G50 sephadex spin column equilibrated with 50 mM Tris/1 mM EDTA (pH 8.0). The labeled oligonucleotide was then annealed to its complementary oligonucleotide by cooling from 90°C to 4°C overnight. The double-stranded oligonucleotide was purified on an 18% polyacrylamide gel. Gel slices containing the template were excised and extracted with Tris-EDTA (pH 8.0) overnight at room temperature.

3'-Extended Oligonucleotide Templates

5'-labeled double-stranded oligonucleotides were extended at their 3' end by incubation with 50 U of terminal transferase (Gibco) in presence of 50 μM dATP in 100 μl reaction consisting of 100 mM potassium cacodylate (pH 7.0), 1 mM CoCl₂, and 0.2 mM DTT, for 30 min at 30°C. After heat inactivation for 10 min, the reaction mixture was passed through a G50 Sephadex spin column.

Exonuclease Activity Assay

Exonuclease activity assay was modified from (Paull and Gellert, 1999). Briefly, 100 μ l of interphase extract was incubated with 1 μ g of digested plasmid or with 1 μ g of digested plasmid and 5 mM caffeine for 30 min at 20°C. The extracts were then diluted 5-fold in PBS 0.1% Triton X-100 and incubated for 2 hr with 25 μ l of Protein A-sepharose 4B (Pharmacia), coupled to 100 μ l of anti-X-Mre11 serum. The beads were washed extensively in buffer containing 0.5 M NaCl, 0.5% Triton X-100, 40 mM Tris (pH 7.4), and 2 mM DTT. The final washes were performed in the reaction buffer consisting of 25 mM MOPS (pH 7.0), 150 mM KCl, 10% polyethylene glycol, 2 mM MgCl₂, 2 mM MnCl₂, and 2 mM DTT. The reaction buffer was brought to 37°C and added to 25 μ l of control beads or to 25 μ l of immunoprecipitates in a 40 μ l reactions containing 0.08 pM of the 5'-labeled double-stranded oligonucleotide or the same double-stranded oligonucleotide with the 3' homopolymeric tail. Reaction mixtures were incubated at 37°C for 60 min. Exonuclease digestions were complete: internal labeling of the oligonucleotide with dNTPs or end-labeling with T4 kinase gave identical results (data not shown). The beads were then collected and the supernatant was incubated with 0.2% SDS, 5 mM EDTA and 0.1 mg/ml proteinase K for 15 min. The reactions were then spotted on DE81 filters (Whatman). The filter were washed in 0.5 M (NH4)HCO₃ and dried under light. Radioactivity associated with undigested double-stranded oligonucleotides on the filters was measured by liquid scintillation counting (Kiss-Laszlo et al., 1996). Some immunoprecipitates from extract treated with digested DNA were incubated with λ phosphatase for 30 min at 30°C, spun again, and washed with high salt buffer and reaction mix buffer.

X-Mre11 Complex Depletion/Cdc6 Depletion

For X-Mre11 complex depletion, 50 μ l of interphase extract were incubated with 25 μ l protein A sepharose beads coupled to 50 μ l preimmune serum or with 50 μ l of X-Mre11 antiserum for 60 min at 4°C. For cdc6 depletion, 50 μ l of interphase extract were incubated with 25 μ l protein A sepharose beads coupled to 10 μ l of cdc6 antiserum (Costanzo et al., 2000) for 60 min at 4°C.

Recombinant Mre11/Rad50/Nbs1 Proteins

Human Mre11/Rad50/Nbs1 complex was purified from baculovirus-infected cells according to published protocols (Paull and Gellert, 1999). The recombinant trimeric complex was used at a concentration of 500 nM, unless specified otherwise.

DNA Replication

DNA replication was performed according to Costanzo et al. (1999). Briefly, nuclei were assembled for 15 min then incubated in interphase extracts at a concentration of 1000 nuclei/ μ l. Aliquots of the reaction were pulsed labeled with α -³²P-dCTP from 0–30 min, 30–60 min, and 60–90 min at 20°C. Reactions were stopped with 5% SDS, 80 mM Tris, 8 mM EDTA (pH 8.0) and digested with 1 mg/ml proteinase K for 30 min. DNA was extracted with phenol/chloroform and electrophoresed on 0.8% agarose gel.

TUNEL Assay

50 μ l of untreated interphase extracts, of extract treated with pre-immune serum or of X-Mre11-depleted extract were incubated with 10,000 nuclei/ μ l for 120 min at 20°C. Extracts were diluted in 1 ml of a buffer consisting of 100 mM KCl, 25 mM HEPES (pH 7.8), 2.5 MgCl₂, and 0.4% Triton X-100. Samples were layered on a sucrose cushion made with the same buffer without Triton and spun for 20 min at 6000 \times g in a HB-6 rotor (Sorvall). The pellet was washed and incubated at 37°C for 4 hr in a buffer containing 90 U of Terminal transferase, 100 mM potassium cacodylate (pH 7.0), 1 mM CoCl₂, 0.2 mM DTT, 25 μ Ci dGTP (3000 cu/mM), and 50 μ M dGTP. Control reactions were incubated in the same buffer but TdT was omitted. Reaction mixtures were then treated with 0.1 mg/ml proteinase K and the DNA was phenol/chloroform extracted and electrophoresed on 0.5% agarose gel at 100 volt for 60 min. The gel was fixed in 20% TCA, dried, and then exposed for autoradiography. The labeled band was excised from the gel and quantified by scintillation counting. 6-DMAP was used at 3 mM concentration, aphidicolin at 100 μ g/ml, and actinomycin D at 10 μ g/ml.

Phosphorylated Histone H2AX Detection

50 μ l of mock-depleted or X-Mre11-depleted interphase extract were incubated with 10,000 nuclei/ μ l for 90 min at 23°C. Postreplicative chromatin was isolated by diluting the extracts in chromatin isolation buffer containing 1 mM NaF, 1 mM Na vanadate, and 0.125% Triton X-100. Samples were layered onto a sucrose cushion in chromatin isolation buffer lacking Triton X-100, then spun at 6000 \times g for 20 min at 4°C. Positive control was prepared by incubating sperm nuclei for 30 min in interphase extract to decondense the chromatin. The chromatin was then isolated and digested for 2 hr with NotI. Digested chromatin was isolated and incubated in interphase extract for 60 min.

Chromatin was boiled in Laemmli buffer and processed for SDS-PAGE electrophoresis. Anti-phosphorylated H2AX antibody was used for Western blot at 1/6,000 dilution.

Checkpoint Assay

X-Mre11 checkpoint function was assayed as previously described (Costanzo et al., 2000), except that M and B or M* and B* fractions were prepared from X-Mre11-depleted extracts as described previously.

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BIOGRAPHICAL SKETCH

Provide the following information for the key personnel in the order listed for Form Page 2.
Photocopy this page or follow this format for each person.

NAME GAUTIER, Jean	POSITION TITLE Assistant Professor, Genetics and Development / Dermatology		
EDUCATION/TRAINING (Begin with baccalaureate or other initial professional education, such as nursing, and include postdoctoral training.)			
INSTITUTION AND LOCATION	DEGREE (if applicable)	YEAR(s)	FIELD OF STUDY
Lycée Pierre de Fermat Toulouse University, France Toulouse University, France Toulouse University, France University of Colorado Health Sciences Center University of California San Francisco	Baccalaureat Master Ph.D. Dr.Sc. Post-doctoral Post-doctoral	1974 1978 1983 1988 1988-1989 1989-1992	Maths, Physics Biology Developmental Biology Developmental Biology Cell Cycle Cell Cycle

RESEARCH AND PROFESSIONAL EXPERIENCE

10/1979-12/1980: School of Agronomy, Mostaganem (Algeria). Teacher (Biology, Genetics).

10/1984-09/1992: Centre National de la Recherche Scientifique, France. Permanent research scientist position as "Chargé de Recherches I".

01/1987-08/1987: Department of Biology, Indiana University (Prof. G.M. Malacinski). Visiting Scientist.

02/1988-12/1989: Department of Pharmacology, University of Colorado Health Sciences Center (Prof. James Maller). Postdoctoral Fellow.

03/1989-06/1989: EMBO short-term fellowship, University of Oxford (Prof. P. Nurse).

12/1989-06/1992: Department of Biochemistry and Biophysics, University of California San Francisco (Prof. Marc W. Kirschner). Postdoctoral fellow. American Cancer Society Senior Postdoctoral Fellow.

06/1992-07/1993: URA 671 CNRS, Station Zoologique, Villefranche sur mer. Directeur de Recherche, CNRS (Centre National de la Recherche Scientifique).

08/1993-08/1995: Roche Institute of Molecular Biology, Associate Member.

09/1995-present: Columbia university, Department of Genetics and Development and Department of Dermatology, Assistant Professor.

HONORS AND AWARDS:

1989: Bronze Medal of the CNRS.

10/1996-09/1997: Mallinckrodt Foundation Award.

01/1997-12/2001: Irma T. Hirsch Scholar Award.

07/1997-06/2001: US Army Breast Cancer Research Program. Career Development Award.

RESEARCH PROJECTS ONGOING OR COMPLETED DURING THE LAST 3 YEARS

"The Functional Role of the Ataxia-telangiectasia Gene". To clone, characterize and study the biochemical role of the ATM protein in G2 checkpoints regulation using Xenopus egg extracts and embryos.

Principal Investigator: Jean Gautier, Ph.D.

Agency: Mallinckrodt Foundation. Period: 10/1/96 - 9/30/99.

“Skin Cancer: UVB-induced cell cycle alterations”. To study the UV-induced G2 cell cycle checkpoints in keratinocytes.

Principal Investigator: David Bickers, M.D.

Co-Principal Investigator: Jean Gautier, Ph.D.

Agency: NIAMSD. RO1. Period: 10/01/98 - 09/30/01

“Cell Cycle Regulation of DNA Replication”. To study the modulation of the assembly and of the function of the pre-replicative complex by cell cycle regulated protein kinases. To design in vitro systems to reconstitute chromosomal DNA replication in vitro.

Principal Investigator: Jean Gautier, Ph.D.

Agency: American Cancer Society. Period: 01/01/99 - 31/12/01.

“Cell Cycle Control by the cAMP-PKA Signal Transduction Pathway”. To study the role of the cAMP-PKA pathway in regulating DNA replication and in coupling mitosis to DNA replication using Xenopus extracts.

Principal Investigator: Jean Gautier, Ph.D.

Co-Principal Investigator: Max Gottesman, M.D.

Agency: NIGMS. 1 RO1 GM56781-01A2. Period: 05/01/99 - 04/30/03.

PUBLICATIONS (Selected)

Costanzo, V., Robertson K., Bibikova M., Kim E., Grieco D., Gottesman M., Carroll D. and **Gautier J.** (2001). Mre11 protein complex prevents double-strand break accumulation during chromosomal DNA replication. *Molecular Cell*, In press.

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Costanzo, V., Robertson, R., Ying, C., Kim, K., Avvedimento, E., Gottesman, M., Grieco, D., and **Gautier, J.** (2000) Reconstitution of an ATM-dependent checkpoint that inhibits chromosomal DNA replication following DNA damage. *Molecular Cell*, **6**, 649-659.

Athar, M., Kim, A., Ahmad, N., Mukhtar, H., **Gautier, J.** and Bickers, D. (2000). Mechanism of ultraviolet B-induced cell cycle arrest in G2/M phase in immortalized skin keratinocytes with defective p53. *Biochem. Biophys. Res. Comm.* **277**, 107-111.

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